Purification and characterisation of xanthine oxidoreductase from liver

Choudhury, Sharmila

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Submitted by Sharmila Choudhury
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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

This thesis is dedicated to the memory of my father

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Abstract

Xanthine oxidoreductase (XOR) is a molybdoflavoenzyme of great interest, particularly because of its proposed roles in ischaemia-reperfusion injury and a range of other pathological conditions. Xanthine oxidoreductases from bovine milk and rat liver have been widely studied, and more recently, the development of a purification procedure for the isolation of XOR from human milk has allowed this enzyme to be characterised. Human milk xanthine oxidoreductase (HMXOR) has been found to be much less active towards traditional substrates, such as hypoxanthine and xanthine, than its bovine counterpart. Reported purifications of the human liver enzyme show specific activity similar to that of bovine milk, and therefore significantly higher than for human milk enzyme.

The work presented here describes the purification and characterisation of human liver xanthine oxidoreductase (HLXOR), with the use of immunoaffinity methods. XOR was purified from bovine liver to near homogeneity as judged by SDS-PAGE, using both affinity and immunoaffinity means of purification. The immunoaffinity purification was then optimised for the isolation of XOR from human liver. Affinity-purified polyclonal and monoclonal anti-(HMXOR) antibodies were characterised for this purpose and the latter were judged to provide better purification, as evidenced by SDS-PAGE. Monoclonal immunoaffinity purification resulted in HLXOR that was homogeneous on the basis of SDS-PAGE and Western blotting, displaying a major band at 150 kDa and a minor band at 130 kDa. This preparation was shown to have a specific activity towards xanthine of 170 nmol/min/mg, which is significantly lower than that reported by other workers, and more comparable to that of the milk enzyme.

A monoclonal anti-(HMXOR) antibody was used in the immunofluorescence localisation of XOR in normal and primary biliary cirrhotic human liver paraffin-embedded sections. Strong staining was seen in hepatocytes, bile ductules and some

Kupffer cells. Similar staining was observed in primary biliary cirrhotic liver, with further evidence of atypical bile ductule configuration. The specific activity of XOR was found to be over 4-fold higher in primary biliary cirrhotic liver homogenate than that of normal liver homogenate, suggesting a form of upregulation of enzyme in the diseased liver.

Small human liver XOR crystals were obtained, and these provide a basis for further clarification of the structure of this enzyme.

Abbreviations

AO – aldehyde oxidase

BLXOR – bovine liver xanthine oxidoreductase

BMXOR – bovine milk xanthine oxidoreductase

BSA – bovine serum albumin

CNBr - cyanogen bromide

DMSO - dimethylsulphoxide

DTT – dithiothreitol

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

FAD – flavin adenine dinucleotide

Fe/S – iron-sulphur

FPLC – fast protein liquid chromatography

HLXOR – human liver xanthine oxidoreductase

HMXOR – human milk xanthine oxidoreductase

HXO – human xanthine oxidase

IFN - interferon

Ig – immunoglobulin

IL-1 – interleukin-1

I-R – ischaemia-reperfusion

kDa – kilodalton

Mo – molybdenum

NAD^+ - nicotine adenine dinucleotide, oxidised form

NADH - nicotine adenine dinucleotide, reduced form

NRS – normal rabbit serum

PBC – primary biliary cirrhosis

PBS – phosphate buffered saline

PFR – protein to flavin ratio

PMSF – phenylmethylsulphonyl fluoride

RNA – ribose nucleic acid

ROS – reactive oxygen species

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SO – sulfite oxidase

TMB - tetramethylbenzidine

TNF – tumour necrosis factor

Tris – tris(hydroxymethyl)aminomethane

UV – ultraviolet

XDH – xanthine dehydrogenase

XO – xanthine oxidase

XOR – xanthine oxidoreductase

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1. Introduction

Xanthine oxidoreductase (XOR) is a major enzyme involved in purine catabolism, participating in the terminal reaction of the purine degradation pathway in man, by catalysing the rate–limiting oxidation of hypoxanthine to xanthine, and xanthine to uric acid. The enzyme is a molybdoflavoprotein, containing molybdenum, flavin adenine dinucleotide (FAD) and iron/sulphur (Fe/S) redox centres, belonging to a family of mammalian molybdenum-containing proteins along with aldehyde oxidase (AO) and sulfite oxidase (SO).

Xanthine oxidoreductase (XOR) has been extensively studied for over a century, dating back to 1882, when Horbaczewski observed the catalysis of hypoxanthine to xanthine to uric acid in tissue homogenates (Horbaczewski, 1882). A few years later, it was demonstrated by Schardinger that samples of fresh milk were able to decolourise methylene blue in the presence of formaldehyde (Schardinger, 1902). The enzyme responsible for this activity was originally named Schardinger's enzyme. Over the next two decades, an activity was revealed to be present in cow's milk, similar to that found in tissue homogenates, that had the ability to convert hypoxanthine and xanthine to uric acid in the presence and absence of oxygen (Morgan *et al.*, 1922). This activity was attributed to an enzyme, XOR. A kinetic analysis of the xanthine and methylene blue activity of partially purified XOR was performed by Dixon & Thurlow (1924, 1926),

who showed that the enzyme was able to utilise aldehydes as reducing substrates as well as purines, thereby indicating that Schardinger's enzyme and XOR were the same.

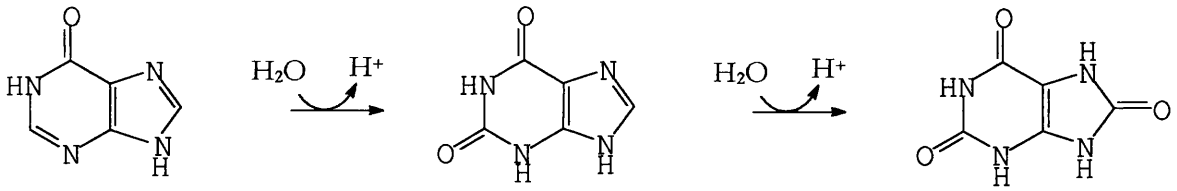
In 1939, an essentially pure preparation of milk XOR was obtained, which possessed a visible spectrum resembling that of a flavoprotein (Ball, 1939). The spectrum indicated the presence of a second chromophore in addition to flavin; this was later determined to be iron (Richert & Westerfeld, 1953). Molybdenum was found to be an additional constituent of the enzyme (De Renzo *et al.*, 1953) making XOR the first mammalian molydoenzyme to be discovered. The ready availability of XOR on a large scale from cows' milk has allowed the pure enzyme to be studied in detail for over sixty years. In particular, the last twenty years have seen enormous advances in structural, sequence, and kinetic information relating to XOR, and molybdoenzymes in general.

XOR is a member of a small family of mammalian mononuclear molybdenum-containing proteins, all of which share a common molybdenum cofactor binding site (Hille, 1996). These molybdoenzymes can be divided into three main categories, namely, the XOR family, the sulphite oxidase (SO) family and the DMSO reductase family. They are unique in that, unlike other biological hydroxylation systems, they utilise water as opposed to molecular oxygen as the source of oxygen atom incorporated into product. Moreover, during turnover, they are able to generate as well as consume reducing equivalents. The hydroxylation reaction typically catalysed is represented below:

$$RH + H_2O \longrightarrow ROH + 2e^- + 2H^+$$

Mammalian XOR is synthesised as xanthine dehydrogenase (XDH) [E.C. 1.1.1.204] and this is the predominant form existing within the cell. However, XDH can be readily converted to xanthine oxidase (XO) [E.C. 1.1.3.22] by oxidation of sulphydryl residues or proteolysis. The enzyme is thus unique within the molybdoenzyme family in that it can exist in either one of two interconvertible forms. These forms have similar reactivities towards reducing substrates, such as xanthine and hypoxanthine, but have different reactivities towards oxidising substrates. XDH shows a preference for NAD^+ whereas XO cannot use NAD^+, requiring molecular oxygen as its substrate.

XOR catalyses the oxidation of hypoxanthine and xanthine to uric acid, and is best recognised for this role as the rate-limiting enzyme in nucleic acid degradation, whereby, purines are channelled for terminal oxidation. The hydroxylation of hypoxanthine to uric acid is accompanied by the concomitant reduction of either NAD^+ to NADH or molecular oxygen to superoxide and hydrogen peroxide, depending on the form of the enzyme, whether dehydrogenase or oxidase.



Hypoxanthine Xanthine Uric Acid

Figure 1.1 **Conversion of hypoxanthine to xanthine and xanthine to uric acid catalysed by XOR**

Mechanism of action

XOR exists as a homodimer with a molecular weight of approximately 300 kDa, composed of two identical sub-units each of 150 kDa. It contains four redox centres per subunit, comprising one molybdopterin cofactor (Rajagopalan & Johnson, 1992), two spectroscopically distinct iron-sulphur (Fe/S) centres (Hille & Anderson, 1991), and one FAD cofactor. Limited proteolysis of the enzyme results in cleavage into three fragments, sequence analysis of which has revealed the molybdenum centre to be located in the C-terminal fragment of 85 kDa, the two Fe/S centres in the N-terminal 20 kDa fragment, and the FAD in the intermediate 40 kDa fragment (Hille & Nishino, 1995). All of these centres have been implicated in catalysis, and a schematic representation of the mechanism of this involvement is shown below:

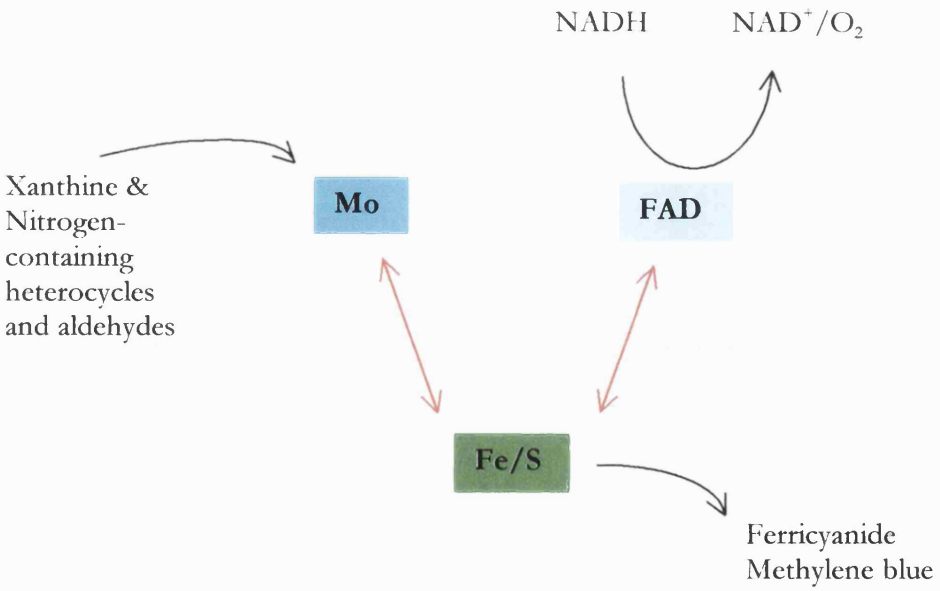


Figure 1.2 Proposed mechanism of action of XOR

Xanthine, and the majority of other reducing substrates, interact with XOR at the molybdopterin centre, reducing Mo(VI) to Mo(IV) (Bray, 1988). Reducing equivalents are subsequently transferred to the FAD centre of the enzyme by intramolecular transfer of electrons via the Fe/S centres (Olson *et al.*, 1974). Reduction of NAD^+ or molecular oxygen occurs through FAD. Molecular oxygen can be univalently or divalently reduced at the FAD site to yield superoxide or hydrogen peroxide respectively (Bray, 1975). These can in turn give rise to secondary radicals including the considerably more reactive hydroxyl radical via the Fenton and iron–mediated Haber–Weiss reactions, as shown below:

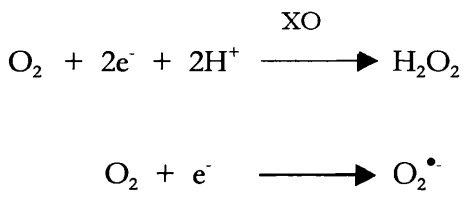


Figure 1.3 **Univalent and divalent reduction of oxygen**

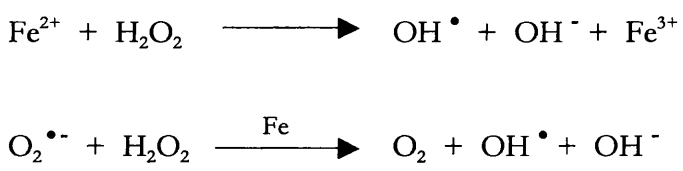


Figure 1.4 **The Fenton reaction and the iron–mediated Haber–Weiss reaction**

This generation of reactive oxygen species (ROS) by the above reactions is well documented, and has led to much interest in XOR as a destructive agent in a range of pathological and physiological conditions, particularly those involving ischaemia–reperfusion tissue injury (McCord, 1985; Sussman & Bulkley, 1990).

It has been shown by spectrophotometric and EPR studies that XOR can accept up to six electrons per subunit (Olson *et al.*, 1974; Bray, 1975). The Fe/S centres are thought to act as an electron 'sink', maintaining the other centres in the optimum redox state (Olson *et al.*, 1974). In addition, they are capable of reducing some artificial substrates such as ferricyanide and methylene blue (Fried & Fried, 1974). As well as xanthine and hypoxanthine, XOR has also been shown to oxidise, by a similar mechanism, a wide range of substrates, including other purines, pyrimidines, pteridines and aldehydes (Massey, 1973; Krenitsky *et al.*, 1986). Despite the broad specificity of the enzyme, it is thought that the high specific activities and low K_M's for hypoxanthine and xanthine identify them as the physiological substrates of XOR.

Both forms of XOR have been shown to catalyse the oxidation of NADH to NAD^+. This NADH-oxidising activity is less widely recognised, although it has been documented for human and bovine milk XOR (Nakamura *et al.*, 1991; Sanders *et al.*, 1997) as well as for turkey and chicken liver XDH (Fhaolain & Coughlan, 1976). NADH donates electrons at the FAD site, in contrast to the other reducing substrates. The oxidation of NADH is catalysed significantly faster by the dehydrogenase form than by the oxidase form of the enzyme and, in both cases, reduction of molecular

oxygen occurs to generate superoxide anion. The NADH oxidising activity is unaffected by all conventional inhibitors which act at the molybdenum centre, such as allopurinol and oxypurinol (Moorhouse *et al.*, 1987), amflutizole (Werns *et al.*, 1991) and BOF-4272 (Uematsu & Nakashima, 1994). The activity is, however, blocked by conventional flavin site inhibitors such as diphenylene iodonium (Robertson *et al.*, 1990). Human milk XOR possesses very low activity to xanthine and hypoxanthine (Abadeh *et al.*, 1992); a fact attributable to its low molybdenum content (Bray *et al.*, 2000). Its NADH oxidase activity is, however, similar to that of the bovine milk enzyme, consistent with the involvement of the FAD site in this reaction. It has been suggested that the 'unimpaired' NADH oxidase activity with regard to the human enzyme reflects a physiological role (Harrison, 1997).

A more recently recognised and much less publicised role for XOR is its capacity to reduce nitrates and nitrites to nitric oxide under anaerobic conditions (Godber *et al.*, 2000a & b; Doel *et al.*, 2000, 2001; Godber *et al.*, 2001), a property perhaps unsurprising in view of the many structural and catalytic similarities XOR shares with plant nitrate reductases (Hille, 1996).

Inhibitors of XOR can be categorised into two groups; those that are structural analogues of purine substrates, and those that are not structurally related to the physiological substrates. Well-known structurally similar inhibitors are allopurinol and oxypurinol (Moorhouse *et al.*, 1987), the former of which undergoes oxidation to produce the latter, which binds tightly to the reduced form of XOR (Massey &

Edmondson, 1970; Robins *et al.*, 1985). Less structurally similar molecules include amflutizole (Werns *et al.*, 1991) and BOF-4272 (Okamoto & Nishino, 1995). All of these inhibitors act at the molybdenum site of the enzyme, and, as noted above, do not affect NADH oxidation.

Xanthine oxidase (XO) and xanthine dehydrogenase (XDH)

Mammalian XOR exists *in vivo* predominantly as XDH, although it may be converted to XO. Both of these species have a similar K_M for xanthine (Waud & Rajagopalan, 1976), however, they differ in terms of electron acceptor. XDH is characterised by a high xanthine to NAD^+ activity, whereas XO is characterised by a high xanthine to molecular oxygen activity, and is unable to reduce NAD^+. It was first observed in 1969 that XOR was largely present in crude homogenate from rats as the NAD^+-dehydrogenase form, whereas the purified enzyme was predominantly in the O_2-dependent oxidase form (Stirpe & Della Corte, 1969). Della Corte and Stirpe went on to demonstrate that the two forms were the same enzyme, and that conversion of XDH to XO could be reversible or irreversible (Della Corte & Stirpe, 1972).

XDH may be readily converted to XO by a variety of conditions, for example, incubation at 37 °C, storage at -20 °C, treatment with organic solvents and with sulphydryl reagents (Stirpe & Della Corte, 1969). These reversible interconversions were attributed to the formation or breakage of disulphide bonds. XDH contains fourteen free sulphydryls per subunit, of which eight can readily form four sulphydryl bonds, although not all of these are involved in XDH/XO interconversion (Waud &

Rajagopalan, 1976; Saito, 1987; Hunt & Massey, 1992). XO is formed irreversibly by proteolysis, which effects the cleavage of a 20 kDa fragment from the 150 kDa subunit. This fragment is not required for oxidase activity of the enzyme, but appears to be essential for the stabilisation of enzyme conformation, and the proper binding and interaction with NAD^+ (Waud & Rajagopalan, 1976).

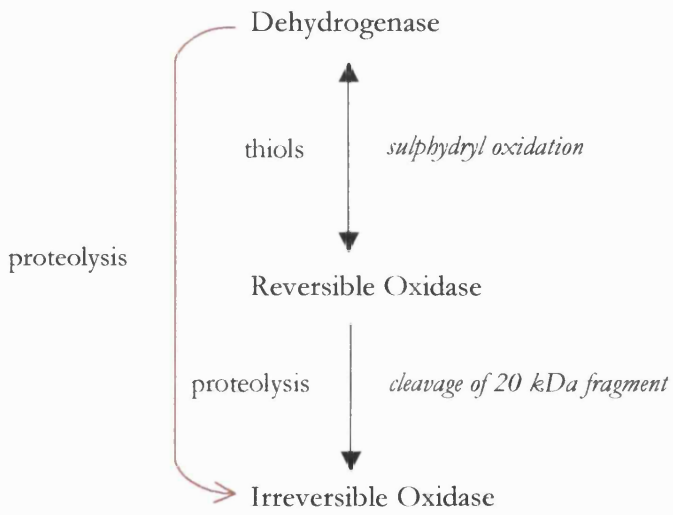


Figure 1.5 Dehydrogenase/oxidase interconversion

Conversion of XDH to XO results in a conformational change in the FAD binding region, involving destabilisation of the anionic forms of the flavin (Saito *et al.*, 1989). In a recent study on the crystal structures of XDH and XO from bovine milk, Enroth *et al.* (2000) observed that cleavage of surface–exposed loops of XDH occurs on proteolytic conversion to XO, causing major structural rearrangement of another loop close to the

flavin ring. This movement prevents NAD binding by partially blocking its access to the FAD cofactor, and thus altering the electrostatic environment of the active site.

Manipulation of sulphydryl reagents allows isolation of bovine milk XOR predominantly in its XDH or XO forms (Nishino *et al.*, 1986; Massey *et al.*, 1989). The two forms of the enzyme are however, encoded by the same gene and can only be easily differentiated by their preference for electron accepting species (Xu *et al.*, 1994; Saksela & Raivio, 1996). There are no distinguishable absorption spectra differences nor are there any marked differences in their K_M values for xanthine (Massey *et al.*, 1989).

Sakuma *et al.* (1997) showed that peroxynitrite can induce reversible conversion of XDH and XO and proposed a metabolic role for this reaction in the liver under certain pathological conditions. It has also been suggested that irreversible conversion of XDH to XO may occur as a result of an uncharacterised proteolytic process taking place in human liver, involving a novel type of proteolytic enzyme that, when triggered by mitochondrial damage, is released from the mitochondria into the cytosol (Saksela *et al.*, 1999).

Inactive forms

Purified preparations of XOR contain inactive forms of the enzyme (Morell, 1952), two of which exist *in vivo*, namely desulpho and demolybdo enzyme.

The desulpho form lacks an Mo=S grouping, essential for molybdenum activity, which is replaced by Mo=O (Bray, 1975). It occurs in enzyme preparations from bovine milk and rat liver (Hart *et al.*, 1970; Ikegami & Nishino, 1986) and makes up approximately 40% of the Mo-containing enzyme in human milk (Godber, B., Ph.D. Thesis, 1998; Bray *et al.*, 1999). Desulpho-XOR can be separated from active enzyme using folate affinity chromatography (Nishino *et al.*, 1981). It can be produced in the laboratory by treatment of reduced enzyme with cyanide (Massey & Edmondson, 1970), which causes the release of sulphur as thiocyanate. This process can be reversed by incubation with sulphide (Massey & Edmondson, 1970; Cleere & Coughlan, 1974; Nishino *et al.*, 1983). Crude and purified rat liver enzyme preparations have been found to consist of up to 40 % desulpho enzyme (Ikegami & Nishino, 1986). Itoh *et al.* (1978) have reported an increase in the specific activity XOR purified from the livers of chickens fed on a diet high in protein, when compared with that of chickens fed on a low protein diet. This increase was attributed to *in vivo* resulphuration of desulpho-enzyme.

Demolybdo-XOR lacks the molybdenum (Mo) atom (Bray, 1975), and to some extent, the associated pterin cofactor. This form of the enzyme makes up some 40 % of bovine milk XOR (Godber, B., Ph.D. Thesis, 1998; Bray *et al.*, 1999). Bray and colleagues have proposed that this percentage reflects dietary intake of Mo. The incorporation of Mo into XOR is one of the final stages in the biosynthesis of the enzyme (Hart *et al.*, 1970; Ventom *et al.*, 1988). Human milk XOR has a very high content (greater than 95 %) of demolybdo-XOR, which explains its low activity towards xanthine and conventional reducing substrates (Abadeh *et al.*, 1992; Godber, B., Ph.D.

Thesis, 1998; Bray *et al.*, 1999). As noted above, NADH oxidase activity of human milk XOR is unaffected by the low Mo content because this activity depends only on the FAD site. Lack of molybdenum and its cofactor may affect the conformation of the enzyme, perhaps accounting for the variation in Fe/S clusters. Bray *et al.* (1999) found that human milk XOR was up to 30 % deficient in Fe/S I, contrasting with bovine milk XOR, which showed little deficiency.

A third inactive form of XOR can be produced in the laboratory but does not appear to exist naturally. This deflavo form lacks FAD and can be produced by treatment of XOR with high concentrations of salt. Its composition of Mo, Fe and sulphur is unchanged. The absorption spectrum, not surprisingly, differs markedly from that of fully active XOR. According to Hille and Massey (1991), it is possible to regain approximately 60 % of the original oxidase activity upon incubation of deflavo XOR with free flavin.

Gene structure and regulation

XOR enzymes isolated from different species show a very high amino acid sequence identity with each other (Glatigny & Scazzocchio, 1995), indicating a high degree of conservation. The mammalian XOR gene sequence is also very similar to that of another molybdoflavoprotein, namely, aldehyde oxidase (AO). Bovine liver AO amino acid sequence shares 50 % identity with that of bovine liver XOR (Li Calzi *et al.*, 1995). Human AO cDNA is 64 % similar to that of human XOR, and the deduced amino acid sequence of human XOR is 49 % homologous with that of human AO (Wright *et al.*,

1993). In addition, the two enzymes possess an overlapping substrate specificity, similar subunit composition and redox centre distribution. It is possible that the two genes arose as the consequence of a duplication event from the same ancestral gene, as they are located on the same human chromosome, separated only by a short distance (Amaya *et al.*, 1990).

AO is a homodimer, consisting of two identical subunits of 150 kDa. The enzyme is expressed at high levels in the liver and small intestine of various animal species (Krenitsky *et al.*, 1974), although levels of AO activity in human tissues appear to be lower than corresponding XOR activities. XOR mRNA is barely detectable in striated muscle and duodenum, whereas significant levels of the AO transcript are present in these organs. In the brain, XOR is present in similarly low quantities, whilst AO again shows differences in regional distribution. It is possible to distinguish AO from XOR on the basis of its greater preference for simple aldehydes and *N*-methylnicotinamide over xanthine. Krenitsky (1978) proposed that XOR and AO together constitute a protective barrier, detoxifying polar aromatic compounds such as nitrogen-containing heterocycles.

The structure of the genes coding for human and mouse XOR have been recently elucidated and found to be very similar (Cazzaniga *et al.*, 1994; Xu *et al.*, 1996), with the exon/intron boundaries of the genes very highly conserved, suggesting the presence of evolutionarily conserved functional domains. The amino acid sequence of bovine milk XOR shows 90 % sequence identity with that of the human liver enzyme (Ichida *et al.*,

1993; Hille & Nishino, 1995; Berglund *et al.*, 1996) and also with those of rat liver (Amaya *et al.*, 1990) and chicken liver (Sato *et al.*, 1995) enzymes. Properties of XOR cloned from various sources are shown in Table 1.1.

The amino acid sequences of XDH from human liver and intestine, traditionally regarded as 'high' activity tissues (Abadeh *et al.*, 1992; Harrison, 1997) have been reported and also found to be essentially identical (Ichida *et al.*, 1993; Saksela & Raivio, 1996; Yamamoto *et al.*, 2001). Recent work has also found the cDNA sequence of human mammary gland XDH, a source of comparatively 'low' activity XOR, to be identical to the other human cDNA sequences (Briggs, 1997; Pearson, A., Ph.D. Thesis, 2001). All the sequences consist of approximately 1,330 amino acids. A weaker homology has been found to exist between the mammalian and *Drosophila* enzymes (Keith *et al.*, 1987; Riley, 1989).

Species	Source	Amino acids	M_r(Da)	Reference
Human	Liver Intestine	1333 1333	146605 146421	Ichida *et al.*, 1993 Saksela & Raivio, 1996
Bovine	Mammary gland	1331	146681	Berglund *et al.*, 1996
Mouse	Liver and spleen	1335	146517	Terao *et al.*, 1992 Cazzaniga *et al.*, 1994
Rat	Liver	1330	146111	Amaya *et al.*, 1990
Chicken	Liver	1358	149613	Sato *et al.*, 1995
Fruitfly (*Drosophila melanogaster*)	-	1335	146925	Lee *et al.*, 1987 Keith *et al.*, 1987
Fruitfly (*Drosophila pseudoobscura*)	-	1342	147422	Riley, 1989

Table 1.1 Properties of XOR cloned from different sources

It has been postulated that there is some degree of post–translational activation of XOR. Evidence for this has been found to exist in *in vivo* systems; XOR has been reported to be upregulated in response to interferon-γ (IFN-γ), in a mouse endothelial cell line (Falciani *et al.*, 1994). Brown *et al.* (1995) suggested presence of hormone control of post–translational activation, which would go some way towards explaining the high initial enzymic activity of XOR in human milk during the first two weeks of lactation, after which activity was shown to decline, an observation not mirrored by a

corresponding decrease in enzyme concentration. Page and colleagues observed that the specific activity of XOR in a human mammary gland epithelial cell line is upregulated by various cytokines, and reported an 8-fold increase in XOR activity. This was accompanied by only a 2- to 3-fold increase in XDH mRNA and protein (Page *et al.*, 1998).

Tissue distribution and cellular localisation of XOR

XOR has been detected in all species studied to date, ranging from mammals to bacteria. Of all mammals studied, XOR has been found to exist in the highest levels in the liver and intestine (Krenitsky *et al.*, 1974). The tissue localisation of XOR in humans has been extensively investigated, particularly since the enzyme has been implicated in the pathogenesis of post-ischaemic reperfusion tissue injury through its ability to produce free radicals (McCord, 1985). However, compared with mammals, XOR activity in man is relatively low (Parks & Granger, 1986). This may reflect major variations in the DNA sequence in the 5' – flanking regions, shown for the rat (Chow *et al.*, 1995), mouse (Cazzaniga *et al.*, 1994) and human (Xu *et al.*, 1996) XOR genes, although this contrasts with the high degree of homology in the coding regions and intron-exon structure as discussed earlier. As noted above, at least in breast milk, human XOR has low Mo content and this is reflected in its low activity. For these reasons, conclusions derived from animal experiments may not directly be extrapolated to humans.

Progress in the development of reliable histochemical methods for the detection of XOR has been hindered by confusion surrounding the properties of the enzyme (Kooij *et al.*, 1991) and the predominance in tissues of the dehydrogenase form; a fact not really realised until 1972 (Battelli *et al.*, 1972). Enzyme activity stains include those based on tetrazolium salts (such as tetranitro blue tetrazolium) (Kooij *et al.*, 1991), or ferri(III)cyanide (Dikov *et al.*, 1988), as electron acceptors. Van Noorden and colleagues (1993) have developed an effective cerium stain, while immunohistochemical methods have been widely used but have suffered from lack of specificity, particularly in the case of polyclonal antibodies (Jarasch *et al.*, 1981; Clare & Lecce, 1991; Hellsten-Westing, 1993; Moriwaki *et al.*, 1996a; Rouquette *et al.*, 1998). The wide range of techniques used in the detection of XOR have led to contradictory results. However, it is widely accepted that XOR is present in the liver and small intestine, as well as the mammary epithelium and capillary endothelium of most tissues.

In cells, XOR is generally understood to be cytosolic, although there have been few extensive published investigations as to its exact subcellular localisation. XOR has been detected in milk-secreting epithelial cells and capillary endothelial cells of the mammary gland, liver sinusoid, heart, lung, intestine and skeletal muscles (Bruder *et al.*, 1983). In rat tissues, liver and renal tubules, as well as the epithelium of the esophagus, small and large intestine and bronchioles stained strongly for XOR, with weaker positive staining detected in the adrenal gland, skeletal muscle, spleen and cerebral hippocampus (Moriwaki *et al.*, 1996a). The detection of XOR in several immune cells (Bruder *et al.*,

1983; Hellsten-Westing, 1993; Moriwaki *et al.*, 1996a) suggests a possible immunological role for the enzyme.

Despite great interest in a possible role of XOR in the pathogenesis of ischaemia-reperfusion damage, there are few reports on the localisation of XOR protein in human tissues (Hellsten-Westing, 1993; Moriwaki *et al.*, 1996b; Linder *et al.*, 1999). Localisation studies of both XOR protein and mRNA are complicated by their close similarity with those of AO, and it is important that detection methods, such as activity assays and antibodies, are carefully characterised. Auscher *et al.* (1980) used radiolabelled xanthine, which detected XOR activity in the epithelial villi of the duodenum and jejunum, but not in the large intestine or rectum. Kooij *et al.* (1992) similarly demonstrated the presence of XOR activity in only the liver and jejunum; findings that were consistent with biochemical results. The precise cellular whereabouts of enzymic activity in the human liver are, however, unclear. Kooij *et al.* (1992) localised XOR activity in sinusoidal cells and in both periportal and pericentral hepatocytes of the liver. Moriwaki *et al.* (1996b) using immunohistochemistry, revealed the presence of XOR in the pericentral and periportal hepatocytes with weaker staining in the mid-zonal area. These workers also found staining in the sinusoidal cells and bile ducts. In contrast, Linder *et al.* (1999), using a similar method of localisation, reported negative staining in the bile ducts and positive staining in Kupffer cells, findings not reported by Moriwaki *et al.* (1996b). They did, however, confirm XOR staining in the cytoplasm of periportal hepatocytes (Linder *et al.*, 1999).

Hellsten-Westing (1993) observed immunoreactivity of XOR in vascular smooth muscle cells, endothelial cells of the capillaries, small vessels in cardiac and skeletal muscle, and in addition, macrophage and mast cells. XOR protein has been shown to be present in the human heart although corresponding enzyme activity is low, and it has been suggested to be largely in an inactive form, similar to that of human milk enzyme (de Jong *et al.*, 1990; Abadeh *et al.*, 1993). Some authors have been unable to detect any activity whatsoever (Downey *et al.*, 1988; Grum *et al.*, 1989). The latter authors concluded that XOR cannot be responsible for the production of harmful oxygen radicals leading to tissue injury in the human heart due to lack of detectable enzyme activity in this organ. It has been suggested that there is a cytosolic inhibitor of XOR, which could account for the apparent lack of enzyme activity in heart (Bray *et al.*, 1959). Hellsten–Westing (1997) demonstrated the presence of XOR in cardiac and skeletal muscle by the use of an immunoaffinity method, and suggested that in view of the overall low or negligible XOR activity in heart tissue homogenates, tissue injury may be initiated at localised vessel sites.

XOR has long been known to be present in relatively high concentrations in milk, where it is associated predominantly with the lipid globule membrane (Patton & Keenan, 1975). As noted above, although amounts of XOR in human and bovine milk are similar, enzyme in human milk has relatively low specific activity, approximately 2 % of that of bovine milk XOR (Jarasch *et al.*, 1986; Abadeh *et al.*, 1992; Harrison, 1997; Sanders *et al.*, 1997). Sarnesto *et al.* (1996) found that the specific activity of human milk enzyme is significantly lower than that found in human liver and intestine.

Circulating XOR

The existence of XOR activity in human serum has long been recognised, although levels have been reported to be very low in normal subjects (Yamamoto *et al.*, 1996). However, several links have been made between disease states and elevated circulatory XOR. Significant increases have been found in incidents of liver (Ramboer *et al.*, 1972; Shamma'a *et al.*, 1973; Giler *et al.*, 1975) and rheumatic (Miesel *et al.*, 1993) diseases and following ischaemia-reperfusion (Friedl *et al.*, 1991; Tan *et al.*, 1993; Trewick *et al.*, 1996). Because of the potential of free circulating XOR to generate harmful ROS, anti-(XOR) antibodies present in serum are especially relevant as they have the capacity to reduce the extent of such injury via inhibitory binding to the enzyme, or by clearance in the form of immune complexes. Antibodies to XOR have not only been reported to be present in human serum (Oster *et al.*, 1974; Bruder *et al.*, 1984; Harrison *et al.*, 1990; Ng *et al.*, 1990; Lewis *et al.*, 1991), but have also been shown to be elevated in patients who had suffered a myocardial infarction (Harrison *et al.*, 1990). These anti-(XOR) antibodies are suggested to be one of the few recognised examples of naturally occurring and beneficial autoantibodies (Benboubetra *et al.*, 1997). An earlier view was that these antibodies could arise from the ingestion of cows' milk or its products, and Rzucidlo and Zikakis (1979) reported an increase in antibodies to bovine milk XOR with increase in milk consumption.

Possible roles of XOR

The ubiquitous localisation of XOR demonstrated by enzyme studies and immunohistochemistry indicate a multi-functional role of this enzyme. Its ability to

generate ROS and free radicals has led to its implication in the pathogenesis of post-ischaemic tissue injury (McCord, 1985). On the other hand, such species may play a beneficial role in signalling (Moulton *et al.*, 2000) or by exerting anti-microbial effects (Tubaro *et al.*, 1980; Van den Munckhof *et al.*, 1995).

Physiological roles

The earliest physiological role attributed to XOR is the catalysis of the oxidation of hypoxanthine and xanthine to uric acid. This rate–limiting reaction is the terminal step of the breakdown of ATP in the purine degradation pathway in man, whereby uric acid is an excretion product. An additional step in this pathway exists in non-primates, where urate oxidase catalyses the further oxidation of uric acid to allantoin. As a result, humans and most primates have higher uric acid concentrations than other mammals, and it has been suggested that physiological levels of uric acid are sufficient to inhibit XOR activity in man (Tan *et al.*, 1993). Uric acid is a powerful antioxidant (Becker, 1993) and has been proposed to act as a chelator of transition metals such as iron and copper (Chapple, 1997). Uric acid production in man is thought to occur mainly at the sites of the liver and intestine, where XOR has been found to be most abundant (Kelley *et al.*, 1989). Although this is certainly an important role of XOR, the enzyme has also been implicated in numerous alternative roles, based on its tissue-specific distribution and wide substrate specificity.

Xanthinuria, first described by Dent and Philpot (1954), is a disease resulting from a lack of functional XOR. It manifests itself by the accumulation of extracellular

xanthine, sometimes known as xanthine stones. The fact that this disease is not life threatening is interesting, as it suggests that the participation of XOR in the catabolism of purines is not crucial. Gout, which is characterised by excess uric acid accumulation, can be effectively managed by treatment with allopurinol, a well-known inhibitor of XOR, which is able to lower serum uric acid in a dose-related manner.

There is evidence for participation of XOR in iron absorption and mobilisation in the small intestine and liver (Topham *et al.*, 1982a; Cohen *et al.*, 1985), utilising NADH and xanthine as reducing substrates to liberate iron from ferritin. Dietary intake studies on the intestine indicate that ingested iron, which is absorbed almost entirely in the ferrous state, is oxidised to the ferric state by XOR in the mucosal cell, which promotes the transcellular transport of iron. Dietary supplementation of tungsten results in rapid inactivation of XOR in the intestinal mucosa, associated with a concomitant reduction of iron transport to blood (Topham *et al.*, 1982b). Also within the liver, a detoxification role has been attributed to the enzyme, based on its wide substrate specificity, and its ability to rapidly catalyse the oxidation of drugs such as the thiopurines and methylxanthines, as well as being able to oxidise even relatively inefficient substrates that may have been ingested, such as deoxyacyclovir, an anti-herpetic prodrug, to acyclovir (Krenitsky *et al.*, 1986).

XOR may fulfil a microbicidal function in milk, as suggested by Bjorck and Claesson (1979), and based on XOR-catalysed production of hydrogen peroxide and superoxide. The recent demonstrations that XOR can catalyse reduction of nitrates and nitrites to

nitric oxide have rekindled interest in a microbicidal role for the enzyme, in the mammary gland, and the neonatal gut (Godber *et al.*, 2000a, 2000b, 2001; Doel *et al.*, 2000, 2001).

ROS are increasingly recognised as cell signalling molecules, thereby implicating the enzyme in signal transduction processes (Khan & Wilson, 1995; Blake *et al.*, 1997). In endothelial cells, XOR is localised extracellularly on surfaces apposing those of other closely neighbouring cells (Rouquette *et al.*, 1998), implying a role in cell-cell interaction, perhaps through ROS. ROS are also known to participate in cell differentiation, metabolism of xenobiotics and the recruitment and activation of polymorphonuclear leukocytes (Granger *et al.*, 1981).

XOR may be involved in a cascade of regulators in the inflammatory process (Harrison, 2000). Interferon-γ (IFN-γ), an inflammatory cytokine, and some IFN-γ inducers, have been demonstrated by several groups to upregulate expression and activity of XOR in various mammalian cell lines (Ghezzi *et al.*, 1984; Dupont *et al.*, 1992; Falciani *et al.*, 1994; Pfeffer *et al.*, 1994). More recently, it has been found that tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1), result in the upregulation and post-translational activation of XOR (Page *et al.*, 1998). These cytokines appear to target endothelial cells, in a process by which the cells respond by upregulating cell adhesion molecules. XOR-derived ROS have been proposed to mediate the upregulation of such transcription factors, which in turn mediate molecules involved in neutrophil adhesion, and thus may play a significant role in inflammatory processes. Lipopolysaccharide, bacteria and

protozoa have been reported to have a similar enhancing effect on XOR activity (Tubaro *et al.*, 1976; Carpani *et al.*, 1990).

Pathological roles of XOR

Proposed pathological roles of XOR primarily involve production of ROS. XOR-derived ROS have been implicated a wide range of pathological conditions, particularly in inflammatory disorders. Examples of such conditions include respiratory distress syndrome, endotoxemia, myocardial infarction, hypertension, rheumatoid arthritis, atherosclerosis and ischaemia-reperfusion (I-R) injury (Granger *et al.*, 1986; McCord, 1987; Sussman & Bulkley, 1990; Winrow *et al.*, 1992; Zweier *et al.*, 1994; Burton *et al.*, 1995).

Ischaemia – reperfusion (I-R) injury

I-R injury is characterised by tissue damage caused by the re-oxygenation of tissues, or reperfusion, that have been subjected to a period of oxygen deprivation, or ischaemia (McCord, 1985). Ischaemia is a widely occurring event, which usually takes place during surgery, under conditions of shock and stress or following organ transplantation. A mechanism for I-R injury was first proposed by Granger *et al.* (1981), and involves ROS, generated from XO after a period of ischaemia. According to this hypothesis, the following sequence of events occurs. During an ischaemic event, tissues are unable to maintain energy production, leading to a net catabolism of ATP, in turn, resulting in an accumulation of cellular hypoxanthine. As the ATP levels drop, the cells lose their ability to maintain low intracellular calcium levels, which are dependent upon the

Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase, resulting in an influx of calcium. The high levels of calcium stimulate calmodulin-dependent activated proteases, which are thought to trigger conversion of naturally-occurring XDH to XO. When oxygen is reintroduced, during the reperfusion period, XO proceeds to oxidise the accumulated hypoxanthine, resulting in the concomitant generation of superoxide anion and hydrogen peroxide, which can further react to produce the more reactive hydroxyl radical. The subsequent actions of these ROS are responsible for tissue damage, taking the form of lipid peroxidation at the cellular membrane, and degradation of hyaluronic acid, a constituent of collagen. This hypothesis has formed the basis for most of the recent research on organ preservation in the liver, heart and kidney.

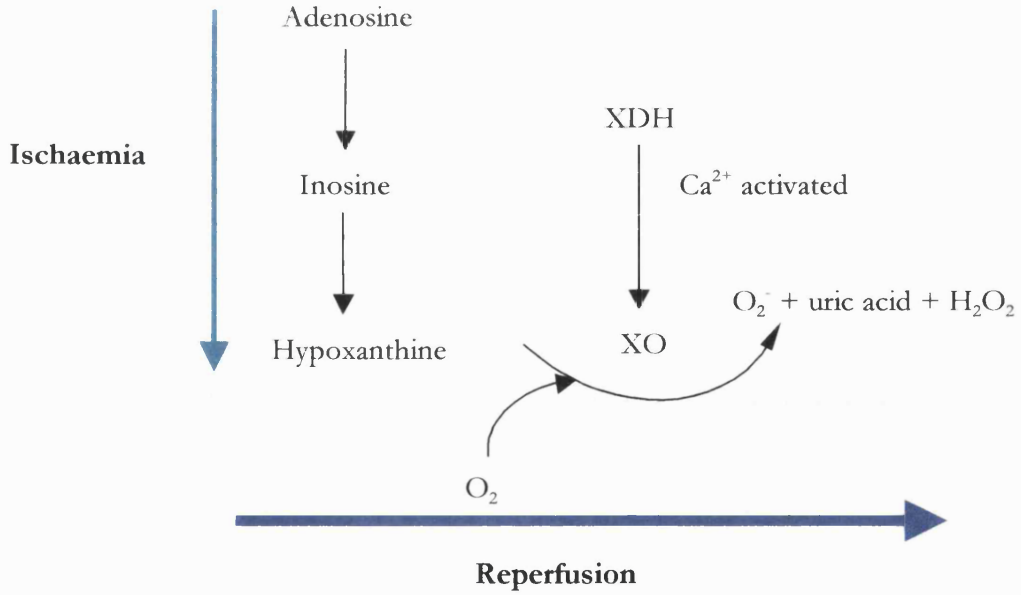


Figure 1.6 **Proposed mechanism for XOR involvement in I-R injury (Granger *et al.*, 1981)**

Several approaches have attempted to alleviate I-R associated injuries. Many investigators have found free radical scavengers or antioxidant enzymes to reduce the extent of reperfusion injury and improve postischaemic function in a variety of tissues, including heart, lung, kidney and brain (Cerutti *et al.*, 1988; Taylor *et al.*, 1989). Recent electron paramagnetic spectroscopy techniques used to measure free radical generation in the ischaemic heart have demonstrated a burst of oxygen free radical generation following postischaemic reperfusion (Zweier, 1988). XOR inhibitors such as allopurinol have been shown to have a positive effect against such injury in both *in vitro* and *in vivo* model systems (Chambers *et al.*, 1985; Charlat *et al.*, 1986). Methylene blue has additionally been shown to have a tissue-protective effect, suggested to be due to its ability to act as an alternative electron acceptor in the catalysis of purines by XOR, thereby inhibiting superoxide formation (Kelner *et al.*, 1988; Salaris *et al.*, 1991).

However, considerable controversy still remains regarding the process by which ischaemia-reperfusion triggers XO-mediated free radical generation (Zweier *et al.*, 1995). Some studies have failed to show conversion of XDH to XO during ischaemia in rat liver (McKelvey *et al.*, 1988; Kooij *et al.*, 1994; Frederiks & Bosch, 1995). One study found that although XDH to XO conversion took place in ischaemic rat liver, this was not followed by a comparable increase in other parameters indicating liver damage, and it was suggested that such XO formation during ischaemia is only a secondary event (Cighetti *et al.*, 1994). Other studies have shown that the XDH-XO conversion proceeds only very slowly during prolonged ischaemia and appears to be a consequence of cellular injury rather than the initiating factor (Engerson *et al.*, 1987; McKelvey *et al.*,

1988; Gonzales-Flecha *et al.*, 1993; Brass, 1995). It has been postulated that, as a consequence of I-R injury, XDH may be released from intracellular locations into the circulation where the conversion to XO may occur, causing additional tissue or cellular injury (Kooij *et al.*, 1994). Recent data indicate that such circulatory XOR can bind to vascular cells, impairing cell function via oxidative mechanisms (Houston *et al.*, 1999).

A major objection to the involvement of XOR in I-R in the heart is the apparent lack of enzyme in this tissue. The heart has been reported several times to be low in, or devoid of, detectable endogenous XOR activity. Eddy *et al.* (1987) could not detect any such activity in the human heart and concluded that the enzyme is unlikely to be a significant source of ROS in the ischaemic heart. It is possible that superoxide and hydrogen peroxide may be derived from sources other than XOR *in vivo*, a point which has been raised by many investigators based on experimental models expressing both high (Gonzalez-Flecha *et al.*, 1993; Cighetti *et al.*, 1994) and relatively low (Joannidis *et al.*, 1990; Dorion *et al.*, 1993) levels of XOR activity. Such alternative sources of ROS may include the mitochondrial respiratory chain and neutrophil NADPH oxidase, although evidence derived from a knockout mouse study has questioned the significance of the latter in free-radical mediated tissue injury (Kubo *et al.*, 1996). The enzymes cyclo-oxygenase, lipoxygenase and nitric oxide synthase have been cited as additional candidates (Zimmerman, 1995). However, a recent investigation using a combination of immunoaffinity and biochemical methods to measure XOR expression and activity has found that both expression and activity were elevated in the failing rat heart but not in the hypertrophic ventricles, suggesting a possible role of the enzyme in the transition from cardiac hypertrophy to failure (de Jong *et al.*, 2000). It is worth noting however,

that this latter data has been obtained in rats, and it is possible that such a role may not apply in humans.

Human XOR

To date, XOR has been purified from only a few human tissues. An affinity purification of XOR from human liver (Krenitsky *et al.*, 1986) was reported to have specific activity very similar to that of XOR from bovine milk. Activity and enzyme-linked immunosorbent assay (ELISA) studies of small intestine also showed relatively high XOR activity (Sarnesto *et al.*, 1996), although the pure enzyme has not been isolated from this tissue as yet.

Abadeh *et al.* (1992) purified XOR from breast milk, which showed a very low specific activity towards conventional reducing substrates, yet shared similar physical properties with bovine milk XOR. This lack of activity was shown to be due to the high proportion of inactive demolybdo and desulpho forms of XOR in human milk (Godber *et al.*, 1997; Bray *et al.*, 1999). An analogous pattern of low activity was also observed in XOR immunopurified from human heart (Abadeh *et al.*, 1993), leading to the suggestion that tissue-specific isoforms of XOR might exist, accounting for such disparity in XOR activity (Harrison, 1997). An alternative explanation might include some form of post-translational activation, for example, resulphuration, as a means of regulation of XOR activity.

2. Aims

XOR from bovine milk fat globule membrane has been intensively studied for many years, and in its pure form for over five decades. Its physicochemical properties have, accordingly, been explored in detail. Significantly less is known about the human enzyme. XOR from the breast milk fat globule membrane has been purified and characterised, showing surprisingly low specific activity to xanthine and related substrates. In contrast, the less-studied enzyme from liver appears to display a relatively high specific activity to xanthine.

In view of indications that the activity of human XOR is variable and tissue-specific, the present work aimed to develop a purification procedure for the isolation and the subsequent characterisation of XOR from human liver. A procedure so-developed could be applied to other human tissues, which, like liver, contain many more proteins than does the milk fat globule membrane. A primary aim of this work was to use rabbit polyclonal antibodies against the human milk XOR as a tool in the affinity purification of enzyme from bovine liver, which could be used as a model for the purification of XOR from human liver. Characterisation of anti-(XOR) antibodies, polyclonal and monoclonal, constituted another aim of this thesis, with an investigation into their potential use in histochemical localisation in tissues.

3. General Materials and Methods

3.1 Materials

3.1.1 Chemicals

Bovine milk xanthine oxidoreductase (from buttermilk) was obtained from Biozyme, Blaenavon, Gwent. Bio-Rad protein assay reagent was purchased from BioRad, Hemel Hempstead, Hertfordshire. DL-dithiothreitol (DTT) was obtained from Alexis Corporation. Sephadex G25 PD-10 buffer exchange columns were obtained from Pharmacia, Uppsala, Sweden. All other chemicals were obtained from Sigma - Aldrich, Poole, Dorset, unless stated otherwise.

3.1.2 Column chromatography matrices

Sephadex G-25 in the form of a PD-10 prepacked column was obtained from Pharmacia Biotech, Uppsala, Sweden.

3.1.3 Instruments

SDS-PAGE was performed using a Bio-Rad mini protean II electrophoresis cell, and Western blotting was carried out using a Pharmacia Biotech nova blot apparatus. Absorbance spectra, Bio – Rad protein estimations, urate and NADH oxidase enzyme assays were performed on a Cary 100 Bio UV-Visible Spectrophotometer.

Fluorescent pterin assays were measured using a Perkin Elmer luminescence spectrometer LS50B. ELISA plates were read on a Tecan Spectra plate-reader (Rainbow Thermo).

3.2 General Methods

3.2.1 SDS-PAGE and Western blotting

3.2.1.1 SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli (1970), using vertical slab gels. Samples to be electrophoresed were diluted 1:1 with reducing sample buffer (62 mM Tris, pH 6.8, 10 % ($^{v}/_{v}$) glycerol, 2 % ($^{w}/_{v}$) SDS, 5 % ($^{v}/_{v}$) β-mercaptoethanol, 1.25 % ($^{w}/_{v}$) bromophenol blue), vortexed briefly then boiled for 3 min. Separating gel was made up of distilled H_2O, 10 % ($^{v}/_{v}$) acrylamide, 2.67 % ($^{v}/_{v}$) N'N bis methylene acrylamide, 375 mM Tris, pH 8.8, 0.1 % ($^{w}/_{v}$) SDS, 0.05 % ($^{w}/_{v}$) APS and 0.05 % ($^{v}/_{v}$) TEMED (NNN'N'-tetramethylethylenediamine). The gel was left to set, during which the stacking gel was made, consisting of distilled H_2O, 4 % acrylamide, 0.9 % N'N bis methylene acrylamide, 125 mM Tris, pH 6.8, 0.1 % ($^{v}/_{v}$) TEMED. This was layered onto the separating gel and the gel comb placed. Once the stacking gel had set, the well comb was removed and the samples were loaded into the wells. The electrophoresis was carried out at 75 V initially, in a tank containing running buffer consisting of 0.192 M glycine, 0.025 M Tris, 0.1 % ($^{w}/_{v}$) SDS, pH 8.3, diluted 1:10 with distilled H_2O, and once samples had passed into the separating layer, the voltage was increased to 200 V.

Electrophoresis was complete when samples had run off the gel. Once elecrophoresis had been completed, unless used for electrotransfer, the gel was removed and stained with Coomassie blue stain [45 % (v/v) methanol, 10 % (v/v) acetic acid and 0.1 % (w/v) Coomassie Brilliant Blue] for 1 h on a shaking platform, and then destaining solution [5 % (v/v) methanol and 7.5 % (v/v) acetic acid] for 3 h at room temperature to visualise proteins.

Molecular weights were estimated by the construction of a standard curve of log molecular weight (Mr) against the relative mobility (Rf), calculated from the total migration distance of the protein divided by that of the dye front. This was done with the high molecular weight standard markers (Sigma-Aldrich) and the molecular weights of unknowns were read using the curve.

3.2.1.2 Western transfer

Four pieces of extra thick filter paper and a single piece of nitrocellulose membrane, corresponding to the size of the gel were soaked in transfer buffer (20 mM Tris-HCl, containing 150 mM glycine, and 20 % (v/v) methanol, pH 8.3). Two pieces of filter paper were placed on the graphite anode plate of the transfer apparatus, also wetted with transfer buffer, followed by the layer of nitrocellulose, then the separating gel, from which the stacking gel had been discarded, was placed on top of the nitrocellulose. The sandwich was completed with the addition of the remaining two filter papers on top of the gel. The cathode plate, also wetted with transfer buffer was placed on top and the transfer run at 0.8 mA/cm^2 for 2 h. To determine whether the transfer had

been complete, colour (rainbow) markers were used, or the membrane was stained with Ponceau S Red and the gel stained with Coomassie blue to visualise proteins.

3.2.1.3 Immunoprobing

Primary antibodies used: affinity–purified polyclonal rabbit anti–(HMXOR), polyclonal rabbit anti–(BMXOR) (Chemicon International), monoclonal mouse anti-(HMXOR) (clone 1D9D1 in the majority of cases) kindly supplied by Eurogenetics, Belgium. Secondary antibodies: anti–(rabbit IgG) (whole molecule) peroxidase conjugate available from Sigma–Aldrich, Dorset, or mouse immunoglobulins peroxidase conjugate available from DAKO A/S, Denmark.

Following transfer, the nitrocellulose membrane was rinsed with distilled H_2O and then washed with TBS [10 mM Tris, 0.9 % ($^{w}/_{v}$) NaCl, pH 7.4] for 5 min at room temperature on a shaking platform. The TBS was replaced with 2 % BSA / TBS to block non-specific binding sites and the membrane was incubated overnight at 4 °C. Washing was performed in triplicate with TBS-T [TBS containing 0.05 % Tween 20] on a shaking platform at room temperature, for 5 min/wash. The membrane was then incubated with primary antibody [1:1000 dilution in TBS-T and 1 % ($^{w}/_{v}$) BSA] and incubated for 2 h on a shaking platform at room temperature. The membrane was washed three times with TBS-T, and the secondary antibody [1:1000 dilution in TBS-T and 1 % ($^{w}/_{v}$) BSA] was applied. The membrane was incubated for 1 h 30 min on a shaking platform at room temperature. Following incubation, the membrane was washed three times with TBS-T and then finally with TBS. To visualise XOR protein,

the membrane was incubated with 4-chloro-2-napthol [3 mg/ml solution in methanol; 16.6 % ($^{v}/_{v}$)], 83.3 % ($^{v}/_{v}$) TBS and 0.04 % ($^{v}/_{v}$) H_2O_2 [30 % ($^{w}/_{w}$) volume)] in a final volume of 10 ml.

3.2.2 Gel filtration using a Sephadex G-25 PD-10 column

Samples were run through a PD-10 column, prior to further column chromatography or assays, for buffer exchange purposes and the removal of low molecular weight endogenous inhibitors, the presence of which might affect assay results. The column was equilibrated with desired buffer (25 ml), and sample was applied in a final volume of 2.5 ml and allowed to run into the column. The same buffer (3.5 ml) was applied to the column and the eluate collected, composing of the larger proteins.

3.2.3 Protein estimation

Total protein content of samples was estimated using the method of Bradford (1976). Bovine serum albumin (1 mg/ml) was used to construct a standard curve ranging from 2-10 µg, diluted with assay buffer to a final volume of 100 µl. Standard and unknown protein samples were added to Bio-Rad protein assay reagent (1 ml) and allowed to develop for 15 min. Absorbances were measured in acrylic cuvettes at 595 nm and protein concentration was calculated from a linear standard curve.

3.2.4 XOR activity assays

3.2.4.1 Pterin assay

The sensitive fluorimetric pterin assay was carried out according to the method of Beckman *et al.* (1989) with a few modifications.

The fluorimeter was used with an excitation wavelength of 345 nm and an emission wavelength of 390 nm with 5 nm band width slits. All assays were performed at 25 °C. Samples were diluted with assay buffer [50 mM potassium phosphate buffer, containing 0.1 mM EDTA, pH 7.4] in 1 ml quartz fluorimeter cuvettes] to a final volume of 1 ml. A stable baseline was first obtained before the addition of substrates. The reducing substrate, 10 μM pterin, was added first, and the cuvette contents mixed by inversion, to determine the oxidase activity of the XOR-containing sample. The production of the fluorescent product isoxanthopterin from pterin, catalysed at the molybdenum site of the enzyme, corresponds with an increase in fluorescence. To assess total activity of XOR, 10 μM methylene blue in addition to the pterin was added. After measuring the resulting reaction rate, the reaction was stopped with the addition of 50 μM allopurinol.

In order to calibrate the reaction and to provide an internal standard accounting for quenching of fluorescence and light scattering of the sample, sequential additions of 10 μM isoxanthopterin (performed five times) were conducted. The reaction rate was calculated and expressed in pmol isoxanthopterin/min/mg total protein. Total protein was estimated by the Bio-Rad protein assay.

3.2.4.2 Urate assay

This assay is a less sensitive method of measuring XOR activity than the fluorimetric method (Section 3.2.4.1). The rate of formation of uric acid from xanthine by the enzyme was followed at 295 nm at 25 °C, using an absorption coefficient of 9.6 $mM^{-1}cm^{-1}$ (Avis *et al.*, 1956). The assay mixture consisted of sample diluted in 50 mM Na-Bicine buffer, pH 8.3, in a 1 ml cuvette to which 100 μM xanthine (100 μl) was added to measure the oxidase form of XOR. 500 μM NAD^+ (100 μl) was then added to the reaction mixture, to provide total activity measurement of XOR. The corresponding dehydrogenase activity was calculated by subtracting the oxidase rate from the rate for total activity.

3.2.4.3 NADH oxidase assay

The NADH oxidase activity of XOR was measured by following the depletion of NADH, forming NAD^+, at 340 nm at 25 °C in air – saturated 50 mM MOPS, pH 7.5. The absorption coefficient used was 6220 $M^{-1}cm^{-1}$ for NADH (Horecker & Kornberg, 1948). The assay mixture consisted of sample diluted in MOPS buffer in a 1 ml acrylic cuvette, to which 50 μM NADH was added, and the resulting decrease in absorbance was followed.

3.2.5 Enzyme – linked immunosorbent assay (ELISA)

3.2.5.1 ELISA for the determination of XOR protein

ELISA kits used for this purpose were kindly donated by Eurogenetics, Belgium. Plates coated with HXO standards were prepared by reconstituting the contents of a vial (containing lyophilised HMXOR) with zero standard/sample diluent (1 ml) to obtain a stock solution of 80 ng/ml. Serial dilutions from this stock were made corresponding to 40, 20, 10, 5, 2.5 and 1.25 ng/ml. Unknown samples were prepared at a range of dilutions in zero standard/sample diluent.

Standards and samples were added to the wells in a total volume of 120 µl, with unknown samples being in triplicate. Plates were sealed and incubated for 120 +/- 10 min at 37 °C. Plates were washed following incubation five times by soaking and inverting with Washing Buffer (400 µl/well, 5 min per wash) at room temperature on a shaking platform.

Biotin–anti– (HXO) conjugate (100 µl) was added, and sealed plates were incubated for 60 +/- 5 mins at 37 °C. Washing was repeated as before. Mixed solution A [citrate – phosphate buffer with peroxide at working strength] and B [3, 3'-5, 5' tetramethylbenzidine solution] (100 µl of each) was added to the wells and incubation was carried out for 20 +/- 2 min at room temperature. Finally Stopping Solution (sulphuric acid, 50 µl) was added to the wells and plates were read at 450 and 650 nm.

3.2.5.2 ELISA for the determination of titre of polyclonal antibodies to XOR

Purified HMXOR was suspended in Coating Buffer [50 mM Na-carbonate, pH 9.6] to a final concentration of 10 μg/ml. The enzyme was added to wells (100 μl/well) and plates were incubated overnight at 4 °C. Wells were washed with PBS-T in triplicate [PBS containing 0.01 % (w/v) Tween 20, 400 μl/well] and then blocked with PBS-T containing 1 % (w/v) BSA (200 μl/well) for 1.5 h at room temperature. Washes were performed as before.

Serial dilutions of normal rabbit serum (NRS) and immune rabbit sera (in PBS-T containing 1 % (w/v) BSA) were performed and these were added to the wells in triplicate. Incubation of the plates for 2 h at 37 °C was carried out. Plates were washed with PBS-T as before.

Secondary antibody [anti–(rabbit IgG) (whole molecule) peroxidase conjugate, diluted 1:5000 in PBS-T containing 1% (w/v) BSA, 100 μl/well) was added and the plates were incubated for 1.5 h at room temperature. Following incubation, plates were washed as described previously, and substrate [1 % TMB in DMSO, diluted 1:100 in 0.5 M Na-acetate-citrate buffer, pH 6, with 30 % (w/v) aqueous hydrogen peroxide added just before use, 100 μl/well]. Colour was left to develop and 0.05 M sulphuric acid (50 μl/well) was added to stop the reaction. After 5-10 min, plates were read at 450 nm.

3.2.6 Aldehyde oxidase (AO) activity assay

Aldehyde oxidase activity in samples was determined at 300 nm, with 1-methylnicotinamide as substrate, at 25 °C, in 50 mM potassium phosphate, pH 7.8, using an extinction coefficient of 4.17 mM^{-1} cm^{-1} (Felsted *et al.*, 1973).

4. The purification of human milk xanthine oxidoreductase (HMXOR)

4.1 Introduction

XOR was first purified from bovine milk in 1939 (Ball, 1939), in a form that was sufficiently pure to provide a visible spectrum, showing the presence of flavin and possibly another chromophore, later discovered to be iron. The bovine enzyme (BMXOR) has since been routinely purified and extensively characterised, because of its relative ease of extraction and its abundance in milk (Bray, 1975). Originally named aldehyde oxidase (Schardinger, 1902), BMXOR has since proved to be a model for the whole class of complex molybdoflavoproteins (Massey & Harris, 1997).

A purification procedure was developed in 1955 by Avis *et al.*, which yielded crystalline XOR. Its UV-visible spectrum gave a protein (280 nm) to flavin (450 nm) ratio (PFR) of between 5.0 and 5.2, which was later accepted to be indicative of a very high level of purity. This procedure utilised a protease (pancreatin), in the initial steps of preparation, which was found to selectively denature and precipitate contaminating casein. Protease treatment was followed by pressure filtration and adsorption on a calcium phosphate column. More reports describing the preparation of pure XOR from bovine milk and other animal sources followed in subsequent years, the majority of which were relatively harsh by current standards, involving heat and acid treatment, drying, solvent extraction and exposure to proteases (Massey & Harris, 1997).

Much of the XOR in milk is reported to be associated with the milk fat globule membrane, thought to be directly derived from the mammary gland secretory cell. Proteolysis has been found to release enzyme from this membrane (Briley & Eisenthal, 1974; 1975). The use of proteases during purification results in a preparation consisting almost entirely of the oxidase form of the enzyme. It is unsurprising, therefore, that not until 1969 was it recognised that there also exists a dehydrogenase form of XOR. Stirpe and Della Corte (1969) reported the apparent activation of rat liver XOR in the presence of proteases, and went on to propose that proteases were able to induce irreversible XDH-XO conversion (see the Introduction section). Nakamura and Yamazaki (1982) found that, by including dithiothreitol (DTT, 2.5 mM) throughout their purification procedure, they could produce XOR consisting of 94 % dehydrogenase form. Upon removal of DTT, reversible conversion to XO occurred; a conversion that they ascribed to the oxidation of sulphydryl groups.

Purification procedures for the isolation of XOR from milk have generally employed centrifugation of whole milk to separate out the cream, followed by butanol treatment and two-step ammonium sulphate precipitation. Various affinity chromatographic steps followed. Calcium phosphate, or hydroxyapatite chromatography, has been widely used and typically gives an increase in purity accompanied by little loss of enzymic activity. Other steps include gel filtration and anion exchange chromatography. A novel affinity chromatographic method was reported by Nishino *et al.* (1981) using folate, a competitive inhibitor of XOR, covalently bound to Sepharose. Folate can only bind molybdenum-containing, or active enzyme, and is therefore also able to resolve active

and inactive forms of XOR. The folate affinity method was based on a previously reported procedure in which an allopurinol analogue, used also as an affinity ligand, was shown to facilitate separation of active forms of XOR (Edmondson *et al.*, 1972). This supported the earlier claim by Morell (1952) that XOR preparations contain non-functional enzyme.

There have been few reports on the purification of human milk XOR (HMXOR), largely because activity assays led to the conclusion that it was essentially absent in this source. Zikakis and McGinnis (1987) obtained a crude enzyme preparation from human colostrom using a combination of salt precipitation, batch-wise hydroxyapatite chromatography, gel filtration and anion exchange chromatography. Their preparation had a molecular weight of 322 kDa and displayed multiple bands on SDS-PAGE gel, along with evidence of aggregation. Graham *et al.* (1989) purified HMXOR, by using salt precipitation followed by hydroxyapatite chromatography, for the purpose of raising antibodies. Their preparation was contaminated by caseins, and as it had a molecular weight of 120 kDa, as judged by SDS-PAGE, it was very likely proteolysed. Hellsten-Westing (1993) purified HMXOR in a similar manner using salt precipitation followed by anion exchange chromatography, also for the purpose of raising antibodies. Again, their purified enzyme was not characterised, and its purity was not mentioned. Abadeh *et al.* (1992) obtained relatively pure HMXOR, using a method based on that of Nakamura & Yamazaki (1982) used for BMXOR purification. This procedure involved solubilisation of enzyme with butanol, and ammonium sulphate precipitation, followed by calcium phosphate chromatography. Yields of enzyme were comparable with those

obtained from bovine milk, having a PFR of 5.13, and indicating a high degree of purity. This method was developed further within our laboratory with the replacement of the calcium phosphate chromatography step (which rapidly lost efficiency with use) by heparin chromatography (Sanders *et al.*, 1997). This followed the findings of Adachi *et al.* (1993), who demonstrated the binding of HMXOR to heparin in a study of XOR binding to cell surfaces. Subsequently, Fukushima *et al.* (1995) showed that XOR was able to bind to sulphated glycosaminoglycans, through lysine and arginine rich peptides. Kjellen and Lindahl (1991) have reviewed the use of heparin in purification of proteoglycans. In our laboratory, the use of heparin cross-linked to 4 % agarose has allowed consistent purification of both BMXOR and HMXOR, in high yields, and with good recovery of enzyme activity.

4.2 Materials and Methods

4.2.1 Materials

Frozen human milk was kindly donated by mothers in the Special Care Baby Units of the Royal United Hospital, Bath; Southmead Hospital, Bristol; Bristol Royal Infirmary and Princess Margaret Hospital, Swindon. Fresh bovine milk was obtained from a local dairy herd in Claverton, Bath. Heparin-agarose (Type I) was obtained from Sigma-Aldrich, Poole, Dorset. Hitrap Q ion exchange columns were obtained from Pharmacia, Uppsala, Sweden. Syringe filters were acquired from Whatman, Maidstone, Kent. All other chemicals were obtained from Sigma-Aldrich, Poole, Dorset.

4.2.2 Methods

4.2.2.1 Preparation of crude HMXOR

XOR was purified from human milk essentially as described by Godber *et al.* (2000). The method used is mild, causing minimal proteolysis and degradation of the enzyme.

Fresh bovine milk (or thawed human milk) (1500-2000 ml) was centrifuged at 2000 g for 30 min at 4 °C to separate the cream. All subsequent operations were carried out at 4 °C. The cream was resuspended in 5 vols 0.2 M K_2HPO_4, containing 1 mM EDTA and 4 mM DTT, with stirring for 90 min. The suspension was centrifuged at 3000 g for 30 min, and the subnatant was collected. The subnatant was filtered through glass wool

and 15 % ($^{w}/_{v}$) ice-cold butanol was added. Ammonium sulphate (15 g/100 ml) was added slowly to the subnatant with stirring, and stirring continued for 1 h. Centrifugation at 8000 g for 20 min followed and the resulting supernatant was filtered through glass wool. Ammonium sulphate (20 g/100 ml) was then added slowly to the supernatant with mixing, and the suspension was stirred for a further 30 min before centrifugation at 9500 g for 30 min. The resulting brown precipitate was collected and resuspended in a small volume of buffer (25 mM Na-phosphate, containing 1 mM EDTA, pH 7.4). Dialysis against the same buffer (3.5 l) overnight followed. Any remaining precipitate was removed by centrifugation at 10000 g for 60 min, and the supernatant was syringe-filtered through a 0.22 μm membrane prior to heparin chromatography.

4.2.2.2 Heparin chromatography

The crude enzyme preparation was subjected to affinity chromatography on heparin-agarose. It was applied (flow rate 20 ml/h) to a column (1 cm x 9 cm) of heparin immobilised on cross–linked 4 % beaded agarose pre-equilibrated in equlibration buffer (25 mM Na-phosphate, containing 1 mM EDTA, pH 7.4). The column was initially washed with the equilibration buffer, to remove unbound protein, followed by 0.05 M NaCl added to the same buffer (20 ml). Elution of XOR was carried out with 0.4 M NaCl added again to the same buffer. Washing of the column following elution was performed with 1 M NaCl added to the equilibration buffer.

Alternatively, for the purification of BMXOR, 25 mM Na-phosphate, pH 7.4, was replaced by 25 mM sodium MES, pH 6, and elution was performed with 0.25 M NaCl added to this buffer.

4.2.2.3 Ion-exchange chromatography using HiTrap Q

Further purification of milk XOR, if necessary, was effected by ion-exchange chromatography on HiTrap Q. Protein eluted from the heparin column was dialysed into 50 mM Na-Bicine, containing 0.05 M NaCl, pH 8.3, syringe-filtered through a 0.22 μm membrane and applied to an equilibrated Hitrap Q ion-exchange column (5 ml) at a flow rate of 1 ml/min for fast protein liquid chromatography (FPLC). Bound protein was eluted by an increasing salt gradient from 0.05 M NaCl to 0.5 M NaCl in 50 mM Na-Bicine, pH 8.3, over a period of 30 min. Enzyme-containing fractions were pooled and concentrated. Following purification, enzyme was drip frozen into liquid nitrogen and stored at –70 °C until further use.

4.2.2.4 Calculation of protein to flavin ratio (PFR)

In order to assess the purity of samples of enzyme, it was necessary to scan them to obtain their UV-visible absorbance spectra. Spectra were typically measured from 700 nm to 250 nm. The ratio of protein (280 nm) to flavin (450 nm) was calculated as A_{280}/A_{450} to give a convenient measure of purity. XOR concentration was also derived from the spectra, using the extinction coefficient, ϵ_{450} of 36000 M^{-1} cm^{-1}; a value which assumes fully functional enzyme (Bray, 1975).

4.3 Results

HMXOR was purified from frozen human milk, as described in the Methods section. Yields of 10 mg purified enzyme were routinely obtained from 2000 ml milk, following ammonium sulphate precipitation, heparin-agarose chromatography and Hitrap Q ion-exchange chromatography. Typical purification data are shown in Table 4.3.1. Elution profiles from the heparin and Hitrap Q columns are shown in Figures 4.3.1 and 4.3.2 respectively. Typical UV-visible spectrum and SDS-PAGE gels of purified enzyme are shown in Figures 4.3.3 and 4.3.4 respectively.

The purified HMXOR was used for many purposes including activity assays, SDS-PAGE analysis, immunisation of rabbits for the production of antibodies, and subsequent affinity purification of specific anti-(HXO) antibodies from rabbit serum.

4.3.1 Purification table for XOR purification from human milk

Fraction	Volume (ml)	Total activity (nmol/min)	Total protein (mg)	Specific activity (pmol/min/mg)	Yield (%)
Milk	2000	2941	40290	73	100
Resuspended cream	485	2028	7562	268	69
Wash with phosphate buffer	455	1235	824	1499	42
1st ammonium sulphate precipitation	376	1088	327	3327	37
Purified XOR (post Mono Q)	7	382	9.2	41521	13

Table 4.3.1 Purification table

XOR activity was determined using the pterin assay (Section 3.2.4.1). Protein quantification was carried out using the Bradford method (Section 3.2.3).

4.3.2 Elution of HMXOR from heparin column

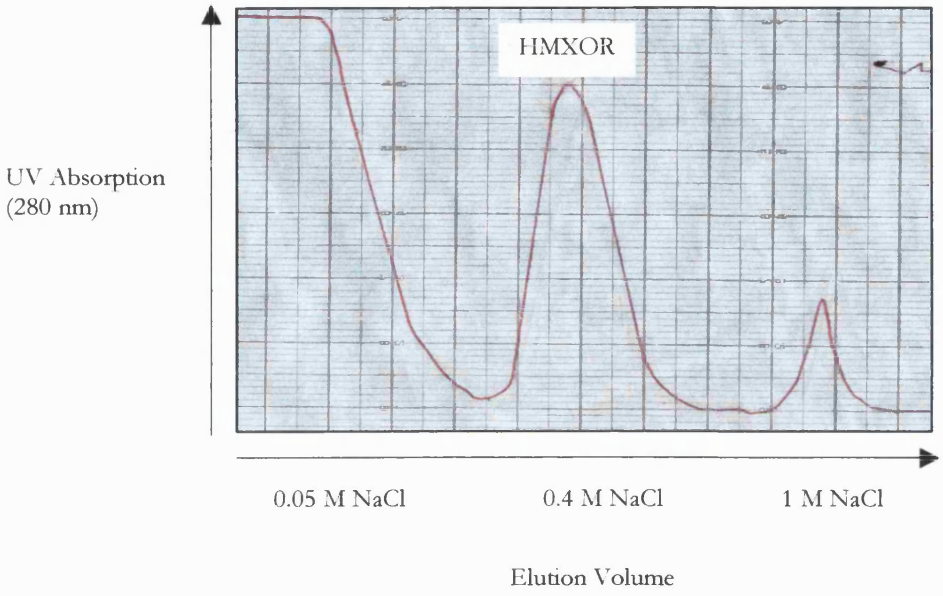


Figure 4.3.1 Elution profile of purification of HMXOR from heparin-agarose column

HMXOR eluted from the column is represented by the middle peak. Elution occurred with 25 mM Na-phosphate, containing 0.4 mM NaCl and 1 mM EDTA, pH 7.4.

4.3.3 Elution of HMXOR from Hitrap Q ion-exchange column

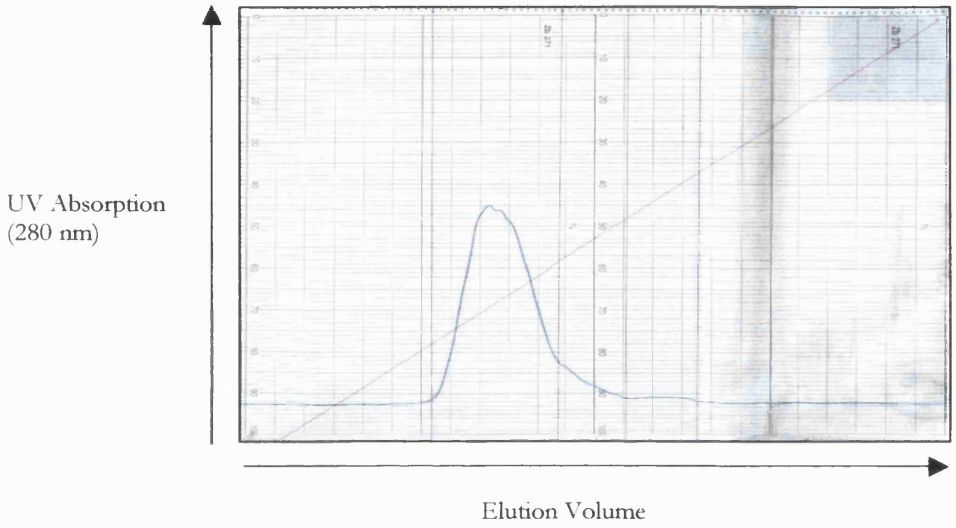


Figure 4.3.2 Elution profile of purification of HMXOR from Hitrap Q ion-exchange column

Straight line (red) indicates the increasing salt concentration (from 0.05 NaCl to 0.5 M NaCl) and the peak on the trace (blue) represents the elution of bound HMXOR.

4.3.4 UV-visible spectrum of purified HMXOR

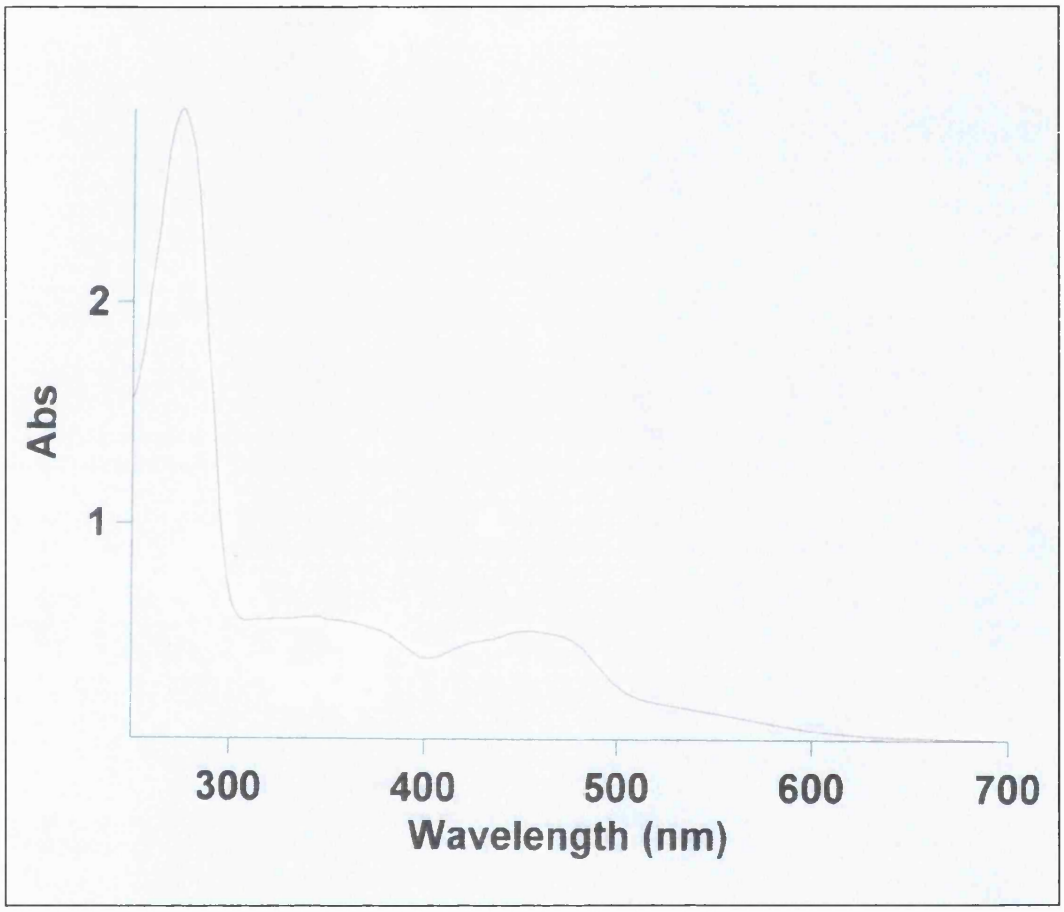


Figure 4.3.3 Typical UV-visible spectrum of purified HMXOR

The above scan represents purified HMXOR after heparin followed by Hitrap Q ion-exchange. Enzyme so prepared typically had a PFR of 5.0 – 5.3.

4.3.5 SDS-PAGE gel of purified HMXOR

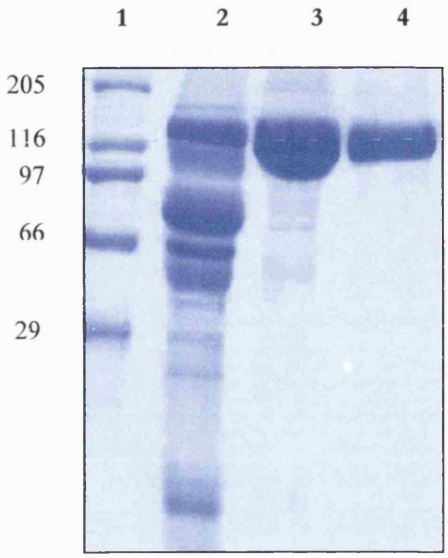


Figure 4.3.4 7.5 % SDS-PAGE gel of purified HMXOR

An SDS-PAGE gel showing in lane 1, high range molecular weight standards, lane 2, ammonium sulphate fraction, lane 3, purified XOR after heparin chromatography, and lane 4, purified XOR after Hitrap Q ion-exchange.

5. The affinity purification of bovine liver xanthine oxidoreductase (BLXOR)

5.1 Introduction

XOR has been isolated and well characterised from bovine milk, rat and chicken liver, and characterised to a lesser extent from human milk. It is known that high levels of XOR are present in the liver and small intestine of all species (Krenitsky *et al.*, 1974), and it has been proposed that XOR and the enzyme aldehyde oxidase (AO), also present in high amounts in the liver, are together responsible for the detoxification of polar aromatic compounds, due to their overlapping substrate specificity (Krenitsky, 1978).

In the organisms studied to date, XDH is the predominant form in mammalian tissues (Nishino, 1994), although mammalian XDH can be converted to XO through sulphydryl oxidation or proteolytic action (Waud & Rajagopalan, 1976). Because XDH-XO conversion is linked to generation of ROS and consequent reperfusion injury, considerable interest has been focussed on the XOR enzymes in different tissues. In order to study them it is necessary to achieve effective purification procedures for their isolation. Ikegami and Nishino (1986) reported a high yield purification of rat liver XOR to apparent homogeneity. Their procedure involved hydroxyapatite chromatography, after which folate chromatography was used to separate out inactive forms of the enzyme. A protein to flavin ratio of between 5.2 and 5.6 was obtained, typical of highly

purified XOR protein. However, this preparation was found to contain both inactive desulpho-XOR and, to a lesser extent, demolybdo-XOR. These inactive forms accounted for up to 50 % of the purified enzyme and a similar level was also found to exist in crude preparations. Ikegami and Nishino (1986) suggested that desulpho-XOR might exist naturally in liver.

A decade later, McManaman *et al.* (1996) reported a novel affinity method for the isolation of rat liver XOR. This group commented that previous affinity methods often involved difficult synthesis and chromatography under anaerobic conditions (Edmondson *et al.*, 1972). They also often involved lengthy procedures, with many chromatographic steps (Ikegami & Nishino, 1986; Abadeh *et al.*, 1992), which may prove deleterious to the enzyme. McManaman *et al.* (1996) developed a purification procedure using immobilised benzamidine as an affinity ligand linked to Sepharose. The structure of benzamidine is similar to that of salicylate, another competitive inhibitor of XOR (Bergel & Bray, 1959). Benzamidine is also a well-known inhibitor of serine proteases, for which it serves as an affinity adsorbent. Furthermore, it has been shown to be an effective inhibitor of aldehyde oxidase (AO), reflecting the overlapping substrate specificity of the two enzymes. The chromatographic procedure employed by McManaman and colleagues was carried out at a pH of 9, and elution effected with 25 mM benzamidine. This step was used in conjunction with heat treatment and ammonium sulphate precipitation as first and second steps respectively. By this method they obtained a near 200-fold purification and an XOR preparation almost free of contaminating proteins.

In contrast to XOR from bovine milk, the enzyme from bovine liver has been little studied. In a histochemical study of bovine liver, XOR was shown to be localised exclusively to the cytoplasm of capillary endothelial cells, with no expression seen in the hepatocytes or Kupffer cells (Jarasch *et al.*, 1981). Current views on the cellular localisation of XOR in humans are mainly based on this study on bovine organs.

One report exists concerning the purification of XOR from bovine liver (Cabre & Canela, 1986). These workers reported a good recovery and outlined some properties of their purified enzyme (BLXOR). Their procedure involved a combination of heat treatment, acetone precipitation, and hydroxyapatite chromatography, followed by several more chromatographic steps. A protein flavin ratio of 5.64 was quoted, and four bands were obtained on SDS-PAGE gel, the largest of which was 135 kDa, these findings probably indicative of a proteolysed preparation. The procedure appeared lengthy, and it has been reported that acetone precipitation can result in chemical modification of the molybdenum centre of XOR (Barber *et al.*, 1976).

This Chapter explores potential methods for the purification of XOR from bovine liver, some of which have been derived from the successful milk XOR purification described in the previous Chapter.

5.2 Materials and Methods

5.2.1 Materials

Fresh calves' liver was obtained from a local butchers in Bath, and kept on ice until ready for use. Benzamidine-Sepharose 4B was obtained from Pharmacia, Uppsala, Sweden. Commercial bovine milk XOR was purchased from Biozyme, Blaenavon, Gwent. Dithiothreitol was purchased from Alexis Corporation. All other chemicals were obtained from Sigma-Aldrich, Poole, Dorset.

5.2.2 Methods

5.2.2.1 Homogenisation of liver and preparation of ammonium sulphate fraction

All operations were carried out at 4 °C, and all equipment was cooled to 4 °C prior to use. Fresh bovine calves' liver (30-50 g) was rinsed in ice-cold PBS, containing protease inhibitors as follows: 5 μM leupeptin, 10 μM chymostatin, 1 μM pepstatin A and 10 μM APMSF, with the addition of 1 mM EDTA and 4 mM DTT. The liver was homogenised in a series of short bursts, using a Waring blender. The resultant homogenate was centrifuged at 5000 rpm, for 30 min. Ground ammonium sulphate was added slowly to the supernatant whilst stirring, to 35 % saturation (19.7 g/100 ml). The suspension was stirred for 1 h. Following stirring, the suspension was centrifuged at 10000 rpm for 35 min. Ammonium sulphate was added a second time to 60 % saturation (15.3 g/ml) and stirred for 1 h. Centrifugation was repeated, at 12500 rpm

for 30 min. The resulting pellet was redissolved in PBS, or the appropriate buffer depending on the chromatography method chosen, containing 1 mM EDTA and 4 mM DTT, and dialysed against 3 l of the same buffer overnight (with two changes). The dialysed ammonium sulphate liver fraction was syringe-filtered using a 0.2 μm disposable syringe filter. Prior to further purification, to remove small molecular weight inhibitors, such as purines, gel filtration was employed, using a Sephadex G-25 PD-10 column (Section 3.2.2).

5.2.2.2 Benzamidine-Sepharose chromatography

This was carried out according to McManaman *et al.* (1996) with a few modifications. Post-ammonium sulphate bovine liver fraction was dialysed against 0.1 M glycine, containing 0.1 M NaCl and protease inhibitors (as in Section 5.2.2.1), pH 9.2. The pH was raised slightly from 9 (McManaman *et al.*, 1996) to 9.2, as this appeared to promote binding.

Benzamidine-Sepharose (5 ml) was poured into a column, and treated according to the manufacturer's instructions. The liver fraction was applied to the benzamidine-Sepharose column equilibrated with 0.1 M glycine, containing 0.1 M NaCl, pH 9.2. The column was washed with 7 vol of this buffer. Weakly-bound protein was eluted from the column with 25 mM benzamidine added to the above buffer, whereas the more strongly bound protein, containing XOR, was eluted with the addition of 50 mM benzamidine. Enzyme-containing fractions were collected in tubes containing 50 mM Na-Bicine, pH 8.3, for neutralisation purposes. Column cleaning and regeneration was

achieved by alternately washing with several column volumes of 0.1 M Tris-HCl containing 0.5 M NaCl, pH 8.5, followed by 0.1 M Na-acetate, containing 0.5 M NaCl, pH 4.5.

5.2.2.3 Heparin chromatography

Heparin chromatography was performed essentially as described in Section 4.2.2.2, for the bovine milk purification. Protein eluted from the benzamidine-Sepharose column was dialysed against 25 mM sodium MES, containing protease inhibitors (as in Section 5.2.2.1), as well as 1 mM EDTA, and 4 mM DTT, pH 6. The dialysed protein was applied to a heparin-Sepharose column equilibrated in the same buffer. Washing of unbound protein was effected with 7 vol of the aforementioned buffer, followed by this buffer containing 0.08 M NaCl to remove weakly bound protein. Elution of more strongly bound enzyme-containing fractions was carried out with 25 mM sodium MES buffer, containing 0.25 M NaCl, pH 6. A final wash to clean the column was carried out using this buffer containing 1 M NaCl.

5.2.2.4 HiTrap Q ion-exchange

HiTrap Q ion-exchange was used as a final step, and this was carried out as described in Section 4.2.2.3 for the purification of XOR from milk.

5.3 Results

5.3.1 The effect of benzamidine on commercial BMXOR

As an initial precaution in the development of a purification procedure to isolate BLXOR, it was necessary to ascertain the effects of eluants on the enzyme. Here, the effects of benzamidine were investigated, using commercially obtainable bovine milk enzyme (BMXOR). The specific activity of BMXOR was measured in the presence of 50 mM benzamidine, in either Buffer A or Buffer B. Buffer A was 0.1 M glycine, pH 9.2. Buffer B was 50 mM potassium phosphate, pH 7.4. This was compared with the specific activity of BMXOR in Buffer B alone.

Conditions of Assay	Specific Activity (pmol/min/mg)
Buffer B alone	38480 ± 5344
Buffer A + 50 mM benzamidine	460 ± 58
Buffer A + 50 mM benzamidine, gel-filtered into Buffer B	27810 ± 3450
Buffer B + 50 mM benzamidine	6260 ± 410

Table 5.3.1 Effect of benzamidine as eluant

XOR activity was measured using the pterin assay (Section 3.2.4.1). Values are means ± SD (n = 3).

The assay showed that BMXOR in the presence of 0.1 M glycine, pH 9.2, containing 50 mM benzamidine, resulted in a greatly reduced activity, which represented approximately 1 % of the original activity. Removal of benzamidine by gel-filtration against 50 mM potassium phosphate, pH 7.4, regained approximately 72 % of the original activity. 16 % of the original activity remained in the presence of 50 mM potassium phosphate, pH 7.4, containing 50 mM benzamidine. This indicates that benzamidine is a more effective inhibitor at the higher pH.

5.3.2 Benzamidine-Sepharose chromatography

Following ammonium sulphate precipitation, bovine liver extract was subjected to benzamidine-Sepharose chromatography, as described in Section 5.2.2.2.

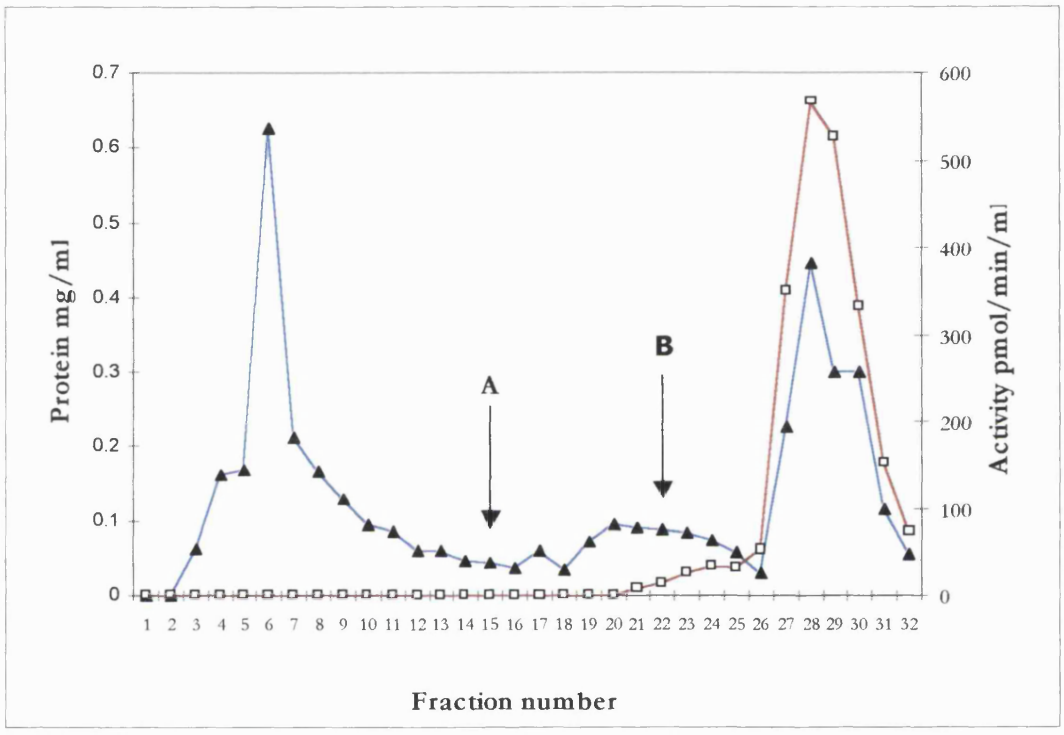


Figure 5.3.1 Elution profile of BLXOR from benzamidine-Sepharose

The above graph shows total protein (mg/ml) represented by the dark blue line, plotted with XOR activity (pmol/min/ml), represented by the red line. Arrow A refers to the addition of 25 mM benzamidine, and arrow B refers to the addition of 50 mM benzamidine. Total protein was estimated using the Bradford method (Section 3.2.3), and XOR activity was measured with the pterin assay (Section 3.2.4.1). XOR activity-containing fractions were eluted from the column with 50 mM benzamidine, and

dialysed into 50 mM MES, containing 1 mM EDTA, and protease inhibitors, pH 6 (Section 5.2.2.1).

5.3.3 Heparin chromatography

The dialysed XOR-containing eluate from benzamidine-Sepharose chromatography was subjected to heparin column chromatography (Section 5.2.2.3).

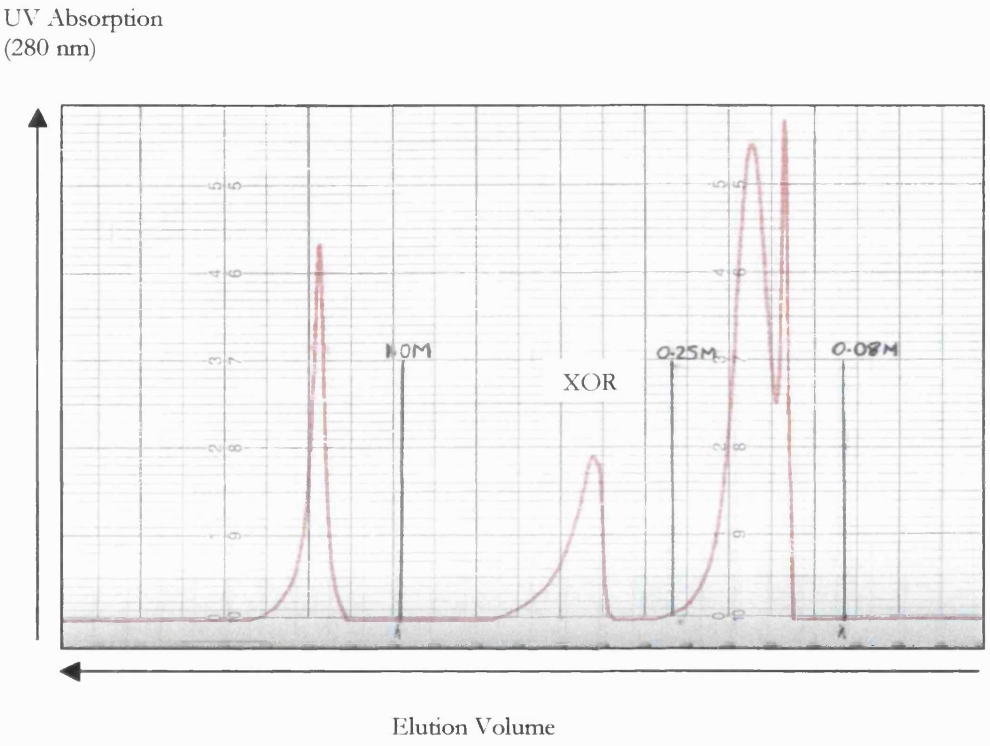


Figure 5.3.2 Elution profile of BLXOR from heparin column

XOR activity was detected in the second major peak only, eluting from the column with 0.25 M NaCl. Resulting XOR activity-containing fractions were pooled and dialysed into 50 mM Na-Bicine, containing 0.05 M NaCl, and protease inhibitors, pH 8.3, in preparation for HiTrap Q ion-exchange chromatography.

5.3.4 HiTrap Q ion-exchange

The dialysed XOR-containing eluate from heparin chromatography was subjected to HiTrap Q ion-exchange (Section 5.2.2.4).

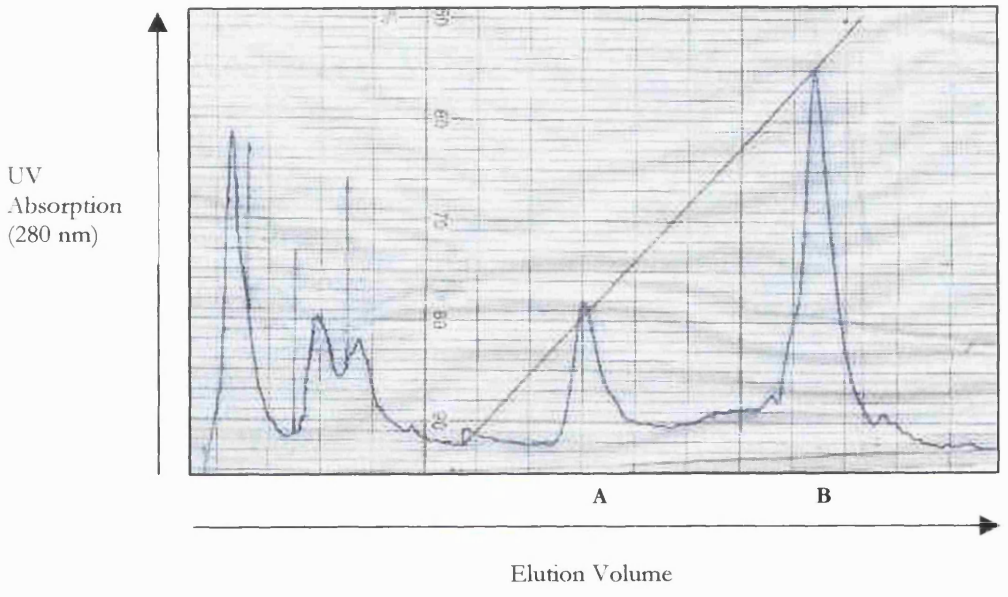


Figure 5.3.3 Elution profile of BLXOR from HiTrap Q ion exchange column

The red diagonal line represents the salt gradient (0.05 M to 0.5 M NaCl). XOR activity was detected in the second major peak (Peak B) only, eluting from the column with 0.4 M NaCl.

5.3.5 SDS-PAGE gel and Western blot

The second major peak (peak B) from HiTrap Q ion-exchange chromatography was run on a 7.5 % SDS-PAGE gel. The purified protein was also subjected to Western blotting using a commercially obtainable anti-(BMXOR) antibody (Chemicon), and was run alongside purified HMXOR.

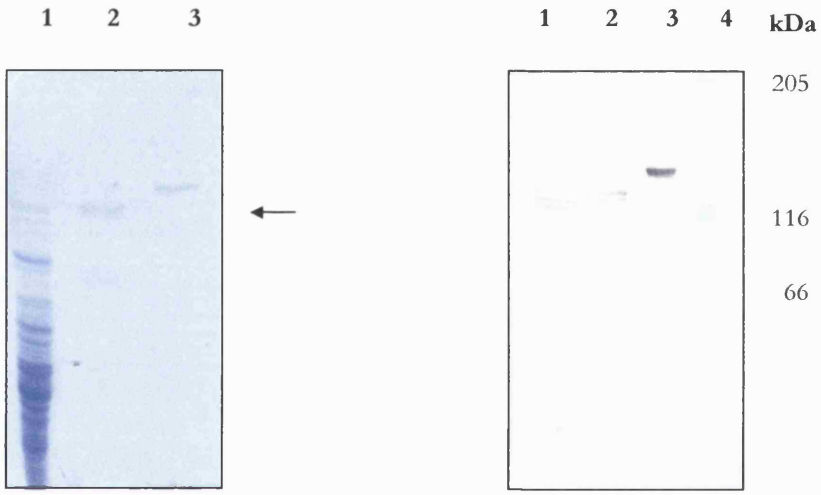


Figure 5.3.4 7.5 % SDS-PAGE gel and Western blot of affinity-purified BLXOR

Lane 1 corresponds to bovine liver homogenate, lane 2 corresponds to affinity-purified BLXOR, and lane 3 corresponds to purified HMXOR. Western blotting was carried out as described in Sections 3.2.1.2 and 3.2.1.3, using a commercial polyclonal anti-(BMXOR) antibody. In the Western blot, lane 1 corresponds to bovine liver

homogenate, lane 2 corresponds to affinity-purified BLXOR, lane 3 corresponds to purified HMXOR, and lane 4 corresponds to high range colour markers.

5.3.6 Specific activity

Purified BLXOR was assayed for activity using the urate assay (Section 3.2.4.2). In order to ascertain that purified enzyme was BLXOR and not AO, which as mentioned earlier also has an affinity for benzamidine, the AO activity assay was performed (Section 3.2.6). A negligible rate was apparent, indicating no contamination of the preparation with AO.

Purification Step	Total Activity (nmol/min)	Total Protein (mg)	Specific Activity (pmol/min/mg)	Fold Purification
Homogenate	2.85	496	6	1
Ammonium sulphate pellet	2.5	27	92	16
Benzamidine pool	1.35	9.4	144	25
Heparin pool	1	1.4	713	124
Hitrap Q eluate	0.16	0.1	1581	275

Table 5.3.2 Purification table for XOR from bovine liver

Protein concentrations were determined using the Bradford method (Section 3.2.3). Specific activities were determined using the urate assay (Section 3.2.4.2).

5.4 Discussion

Using a combination of three chromatographic techniques, namely benzamidine-Sepharose, heparin-Sepharose, and HiTrap Q ion-exchange, the latter two of which are used in the purification of XOR from milk, BLXOR was purified to apparent homogeneity as judged by SDS-PAGE gel. The purified enzyme yielded a major band of approximately 135 kDa. From the SDS-PAGE gel it can be observed that the liver enzyme runs at a slightly lower molecular weight than that of HMXOR. Immunoprobing was carried out with commercial polyclonal anti-(BMXOR) antibody. Strong staining of the 135 kDa band corresponding to BLXOR is apparent as well as a band of slightly higher molecular mass yielded by HMXOR. The overall purification achieved was approximately 275-fold.

Cabre and Canela (1986), who are responsible for the only reported purification of BLXOR, using a combination of five affinity chromatographic steps, with heat and acetone precipitation, obtained BLXOR showing four protein bands on SDS-PAGE gel. These bands corresponded to molecular weights of 135 kDa, 95 kDa, 45 kDa and 15 kDa. This indicates a significantly more proteolysed enzyme than that obtained in the present work, which is represented by a band of approximately 135 kDa, with no additional bands observed upon gel electrophoresis or Western blotting.

The overall purification of 275-fold (Table 5.3.2) achieved in the present work was higher than the 199-fold purification for rat liver XOR obtained by McManaman *et al.* (1996), from which the benzamidine-Sepharose method was slightly modified.

McManaman *et al.* used a combination of heating, ammonium sulphate precipitation and chromatography on benzamidine-Sepharose. Modifications of the present work included raising the pH from 9 to 9.2, which appeared to promote binding (data not shown), and elution with a higher concentration of benzamidine (50 mM). 25 mM benzamidine, as used by McManaman *et al.*, was found to be insufficient to release XOR from the column. This concentration of benzamidine (25 mM) was therefore incorporated as a washing step to remove non-specifically or weakly bound protein. It is likely that differences exist between XOR enzyme from different sources, altering their affinity for benzamidine and possibly also the efficiency of the overall purification. McManaman *et al.* (1996) found that although benzamidine inhibited bovine milk XOR, inhibition only occurred at very high concentrations (estimated K*i* greater than 100 mM), in contrast to rat liver XOR.

A consideration that must be taken into account when using benzamidine chromatography for the purification of XOR is the potential co-purification of AO, which also has an affinity for benzamidine. In the early stages of the development of the purification, AO activity was monitored (Section 3.2.6), and activity was detected in initial liver homogenate but not following the second ammonium sulphate precipitation, of 60 % saturation. AO thus need not pose a co-purification problem when using benzamidine-Sepharose chromatography. In support of this, no AO activity was detectable in the purified XOR preparation.

Heparin chromatography used as a second step provided an almost 5-fold purification from the benzamidine step (Table 5.3.2). The method was essentially the same as for the purification of XOR from bovine milk within the laboratory and different to that used for the purification of human milk XOR. It appears, therefore, that BLXOR and BMXOR express comparable binding affinities for heparin, which differ from those of HMXOR.

Protease inhibitors were included during both benzamidine and heparin chromatography. Benzamidine is a well-known serine protease inhibitor (Hixson & Nishikawa, 1974). Tryptase, a major protein in mast cells and also a serine protease, has been reported to associate with heparin and has indeed been isolated using heparin-Sepharose (Chan *et al.*, 1999). This is of importance, because both benzamidine and heparin bind serine proteases. Precautions have been taken therefore, to ensure that if there are proteases present, these are inhibited and the enzyme thus protected from proteolysis.

HiTrap Q ion-exchange chromatography was performed according to the milk purification procedure (Section 5.3.4). This step was responsible for removing much contaminating protein in the form of both unbound and bound protein, as can be seen from the elution profile (Fig 5.3.3), in which peak A relates to bound contaminants. The combination of these three methods yielded 0.1 mg pure BLXOR from 35 g liver. This compares favourably with McManaman *et al.* (1996) who obtained a yield of 0.13 mg pure rat liver XOR (RLXOR), from a similar starting amount of liver (10-30 g liver).

The purified preparation exhibited a specific activity towards xanthine of approximately 1580 pmol/min/mg (Table 5.3.2). This activity is lower than that generally shown by purified HMXOR (0.3-0.1 μmol/min/mg) (Godber, B, Ph.D. Thesis, 1998). An explanation for this low specific activity might include irreversible inhibition by benzamidine. Treatment of BMXOR with benzamidine produced complete inhibition, yet the majority (72 %) of activity was regained upon removal of benzamidine (Table 5.3.1). However, prolonged incubations of enzyme with benzamidine and specific activity measurements were not carried out. In addition, it was not possible to carry out inhibition studies on the liver enzyme in its pure form, on which the inhibitor may have a more detrimental effect than on the milk enzyme.

Cabre and Canela (1986) obtained a protein flavin ratio from their purified bovine liver preparation corresponding to 5.64, although the absorption spectra from which this was calculated was not shown. Although it was attempted to acquire an absorption spectra from the purified enzyme described in this Chapter, the curve did not resemble the typical spectra produced by XOR, therefore it was unfeasible to calculate a protein flavin ratio in this instance. Reasons for the presence of an atypical spectrum may include proteolysis of the enzyme as a result of purification, a feature much commented upon in previously reported purifications, or minor contamination, not apparent on SDS-PAGE gel analysis, 'masking' absorption of flavin and iron-sulphur centres, both of which are responsible for the characteristic peaks at wavelengths of approximately 350 nm and 450 nm.

6. The purification of polyclonal anti-(HMXOR) IgG

6.1 Introduction

In order to clarify the pathophysiological role of XOR in humans it is vital to investigate its localisation and distribution in tissues, and anti-(XOR) antibodies have proved to be a useful tool in immunohistochemical studies. Many studies have utilised purified polyclonal antibodies to XOR, from both human and bovine sources, in order to elucidate the localisation of XOR in a range of bovine (Jarasch *et al.*, 1981) and human (Jarasch *et al.*, 1986; Moriwaki *et al.*, 1996b; Linder *et al.*, 1999) tissues. Such immunoaffinity detection methods have the advantage over activity staining in that they do not distinguish between active and inactive forms of the enzyme and are thus able to give a more accurate overall representation as to the distribution of protein in tissues.

The use of anti-(XOR) antibodies in immunoaffinity-based purification provides a potentially selective and powerful tool for the rapid isolation of XOR from tissue sources. Saksela *et al.* (1999) purified human liver XOR, using rabbit polyclonal anti-(XOR) antiserum, for the purpose of proteolysis studies. However, details of their immunoaffinity purification method were not given. Abadeh *et al.* (1993) reported an immunoaffinity purification of XOR from human heart, using polyclonal antibodies to human milk. The resulting heart XOR was found to have very low specific activity towards xanthine (as discussed in the Introduction) consistent with a high proportion of inactive forms.

This Chapter describes the development of an immunoaffinity purification method for the isolation of XOR from liver sources.

6.2 Materials and Methods

6.2.1 Materials

Purified HMXOR was prepared as described in Section 4.2.2, and used for the immunisation of rabbits. Imject Alum adjuvant was obtained from Pierce, Netherlands. All other chemicals were obtained from Sigma-Aldrich, Poole, Dorset.

6.2.2 Methods

6.2.2.1 Immunisation of rabbits

Rabbits were initially immunised with Imject Alum adjuvant, which encourages the induction of a strong antibody response, combined with purified HMXOR (400 μg) in a final volume of 0.5 ml. On day 28 following immunisation, a boost injection of purified HMXOR (400 μg) was administered. High levels of IgG antibodies are produced as a result of the second injection, rather than IgM, of which a substantial proportion is present in the sera from primary injections. On day 35, a test bleed was collected, and the titre of serum anti-(HMXOR) antibodies were determined by ELISA, using normal (non-immunised) rabbit serum (NRS) as a control. Following the boost injection, and once a good titre was obtained, further injections were given routinely every 4-6 weeks, the response to these injections mirroring that of the secondary injection, with the maturation of the immune response and the production of high-affinity antibodies.

6.2.2.2 Serum preparation

Blood was collected from the rabbits (40 ml/rabbit) and allowed to contract overnight at 4 °C. The clot was separated from the sides of the collection vessel using a Pasteur pipette and centrifuged at 3000 g for 15 min. Straw-coloured serum was aspirated from the surface of the clot, and further centrifuged at 10000 g for 10 min to remove any insoluble material. The serum was then aliquoted and stored at –20 °C for later use.

6.2.2.3 Purification of a crude preparation of immunoglobulin

Ammonium sulphate precipitation is a commonly used procedure for removal of proteins from solution. Most rabbit antibodies will precipitate from a 50 % saturated solution. Saturated ammonium sulphate solution was added slowly to the serum while stirring gently, to bring the solution to 50 % saturation. The antibody solution was left stirring overnight at 4 °C. The precipitate was centrifuged at 3000 g for 30 min. The supernatant was removed and discarded, and the pellet was resuspended in an equal volume of PBS, containing 0.01 % ($^{w}/_{v}$) sodium azide. The antibody solution was dialysed at 4 °C against three changes of the same buffer to remove ammonium sulphate, and was then centrifuged or syringe-filtered to remove any remaining insoluble debris.

6.2.2.4 Purification of IgG using Protein A-Sepharose

Protein A, a constituent of the cell wall of *S. aureus*, has a high affinity for the Fc region of rabbit IgG. When linked to Sepharose 4B, it can be used to provide a purification matrix for the isolation of IgG from a heterogeneous antibody solution. Protein A-Sepharose (5 ml) was swollen and poured into a column following the manufacturer's

instructions. Precipitated immunoglobulin (5 ml) (Section 6.2.2.3) was diluted 1:10 in PBS and circulated on the column overnight at 4 °C. The column matrix was washed extensively with PBS until the A_{280} had reached baseline, monitored using an attached UV detector linked to a chart recorder. Bound IgG was eluted from the column with 35 mM diethylamine, containing 35 mM NaCl, pH 11.5. The eluted protein was collected in neutralising buffer (1:5 volume of PBS) and then dialysed at 4 °C against PBS, containing 0.01 % ($^{w}/_{v}$) sodium azide.

6.2.2.5 Preparation of an HMXOR-affinity column

An HMXOR-affinity column was used to specifically purify anti-(HMXOR) IgG from the crude IgG fraction. Purified HMXOR (20 mg, PFR 5–5.5) was coupled to CNBr-activated Sepharose 4B at a ratio of 5–10 mg protein per ml swollen gel (routinely a 2 ml column was prepared). HMXOR was gel-filtered, using a Sephadex G-25 PD-10 column (Section 3.2.2), into Coupling Buffer (0.1 M $NaHCO_3$, containing 0.5 M NaCl, pH 8.3). The required amount of CNBr-activated Sepharose 4B freeze-dried powder was swollen in 1 mM HCl (ice-cold), and washed for 15 min on a sintered glass filter with the same solution. Washing with HCl prevents hydrolysis of the reactive groups, which occurs at high pH. The gel was then transferred rapidly to the HMXOR solution and the suspension left to rotate end-over-end overnight at 4 °C (or for 2 h at room temperature). Following incubation, the gel suspension was centrifuged at 3000 g for 5 min and the supernatant aspirated off. The gel was transferred to blocking buffer (1 M ethanolamine) and left to rotate end-over-end at room temperature for 2 h. Excess, or unadsorbed, protein was washed away with a series of buffers: Coupling Buffer followed by 0.1 M Na-acetate, containing 0.5 M NaCl, pH 4, followed by several final

washes of Coupling Buffer, on a sintered glass filter. The HMXOR-Sepharose 4B protein matrix was poured into a column and stored at 4 °C in 0.01 % ($^{w}/_{v}$) sodium azide until ready for use.

Efficient coupling was checked by assaying initial and final concentrations of protein, using the Bradford method (Section 3.2.3). Routinely, a coupling efficiency of between 95 and 98 % was achieved.

6.2.2.6 Affinity purification of anti-(HMXOR) antibodies

The IgG fraction (40 ml) (Section 6.2.2.4) was applied directly to an HMXOR-affinity column (Section 6.2.2.5) and circulated on this column overnight at 4 °C. Following circulation, the column was washed with PBS until A_{280} reached baseline. Bound protein was eluted with 35 mM diethylamine, containing 35 mM NaCl, pH 11.5, and collected in tubes containing neutralising buffer (1:5 volume of PBS). Dialysis was carried out against three changes of PBS (containing 0.01 % ($^{w}/_{v}$) sodium azide). Regeneration of the column was effected by washing with PBS, followed by 0.1 M Na-acetate, pH 4.5. The column was then stored in PBS containing 0.01 % ($^{w}/_{v}$) sodium azide. After several uses (greater than seven purifications) a new HMXOR-affinity column was prepared.

The purified antibodies were concentrated if necessary using Vivaspin 2ml concentrators (Vivascience), and analysed on SDS-PAGE gel or by ELISA. The antibody solution was diluted 1:1 in glycerol to prolong its lifetime, and stored at – 20 °C.

6.3 Results

6.3.1 Immunisation of rabbits

Two rabbits, designated 256 and 257, were immunised with HMXOR (Section 6.2.2.1) and antiserum was prepared (Section 6.2.2.2). Anti-(HMXOR) antibody levels, also referred to as serum titre, were determined by ELISA (Section 3.2.5.2). Titres of pre-immune sera were similarly determined.

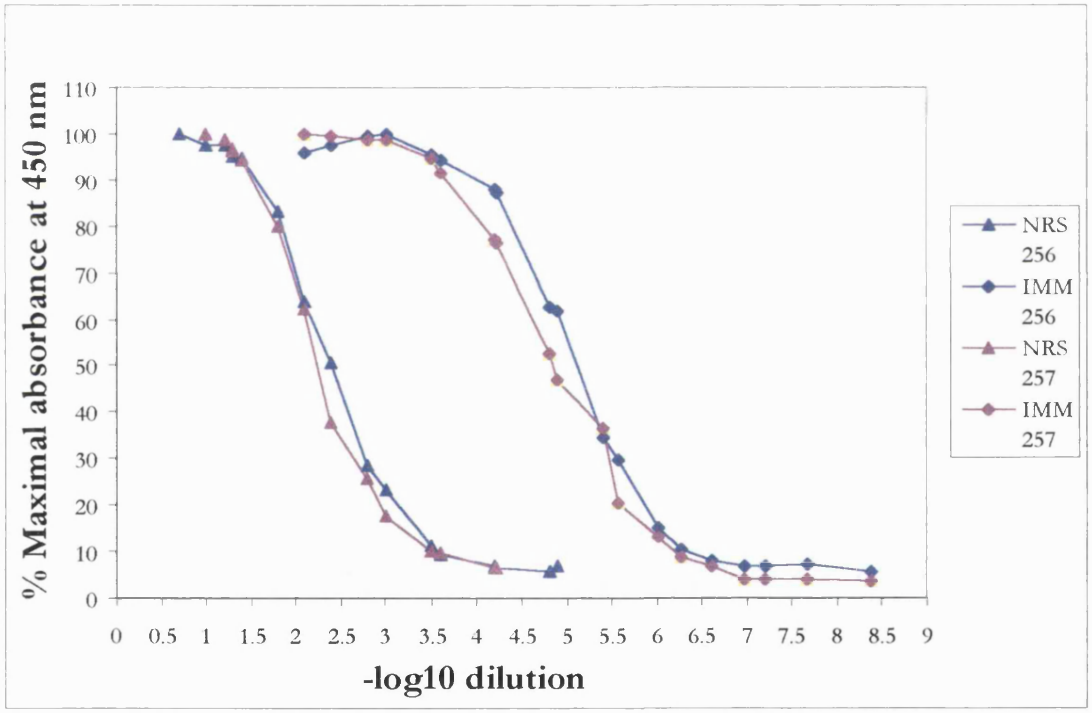


Figure 6.3.1 Anti-(HMXOR) titration curves obtained from rabbit sera

The curves on the left correspond to the sera of the two rabbits (256 and 257) before immunisation (NRS), and the curves on the right following immunisation (IMM).

Titres of the sera from the two rabbits were calculated.

	Rabbit 256	**Rabbit 257**
Normal rabbit serum (NRS)	0.0316	0.0199
Immunised rabbit serum (IMM)	14.8	6.31

Table 6.3.1 Titre of NRS and IMM sera

The values in Table 6.3.1 represent titres x 10^4 derived from the curves in Fig 6.3.1. Titre corresponds to the antilogarithm at 50 % maximal absorbance. Approximate titre increases of 470-fold and 320-fold were obtained following immunisation of rabbits 256 and 257 respectively.

6.3.2 Purification of IgG from precipitated immunoglobulin by chromatography on Protein A-Sepharose

Precipitated immunoglobulin was prepared from rabbit serum (Sections 6.2.2.2 and 6.2.2.3). IgG was purified from precipitated immunoglobulin by chromatography on Protein A-Sepharose (Section 6.2.2.4). The immunoglobulin solution was circulated on the Protein A column until all IgG had been removed, and no change in absorbance was detected at 280 nm.

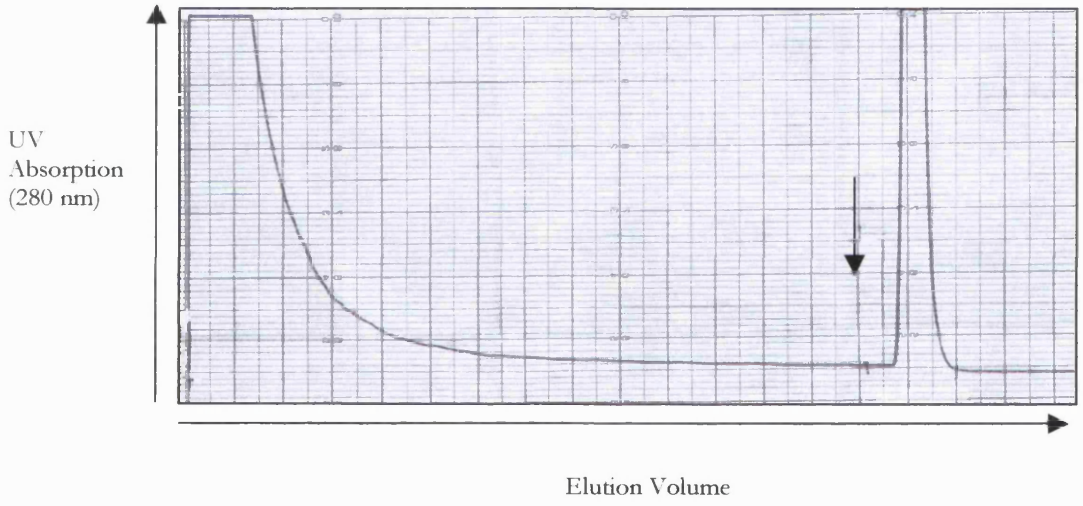


Figure 6.3.2 Purification of IgG from rabbit antiserum

The trace shows the elution of bound IgG from immunoglobulin solution on Protein A-Sepharose. The arrow represents the addition of 35 mM diethylamine, containing 35 mM NaCl, pH 11.5.

6.3.3 Purification of anti-(HMXOR) IgG by chromatography on HMXOR-affinity column

Anti-(HMXOR) IgG was purified from the dialysed rabbit IgG preparation (Section 6.2.2.4) by circulation on immobilised HMXOR (Sections 6.2.2.5 and 6.2.2.6). The IgG preparation was circulated on the column and elutions of anti-(HMXOR) IgG (with 35 mM diethylamine, containing 35 mM NaCl, pH 11.5) were carried out, until absorbance at 280 nm was no longer detectable in the eluant. Usually three circulations were required to remove all specific anti-(HMXOR) IgG. It was observed that the characteristic brown colour due to the immobilised HMXOR was lost from the column during initial usage. With repeated use, the performance of the column declined, and, for this reason, after seven consecutive purifications of anti-(HMXOR) IgG, a new column was prepared.

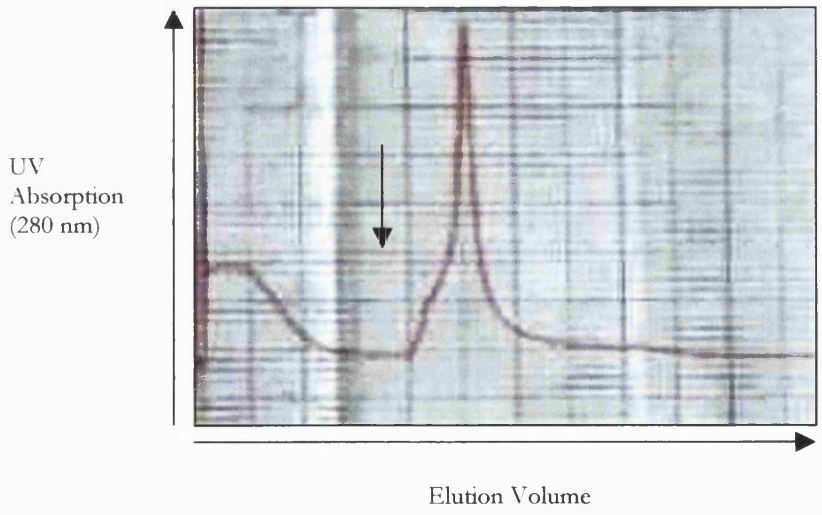


Figure 6.3.3 Purification of anti-(HMXOR) IgG from rabbit IgG

The trace shows the elution of bound anti-(HMXOR) IgG from HMXOR-affinity column. The arrow represents the addition of 35 mM diethylamine, containing 35 mM NaCl, pH 11.5.

The total yield of anti-(HMXOR) IgG purified from rabbit IgG preparation (40 ml) was calculated. Three circulations and consecutive elutions were carried out from the same preparation of IgG.

Rabbit	**Yield of anti-(HMXOR) IgG (mg) from first circulation**	**Yield of anti-(HMXOR) IgG (mg) from second circulation**	**Yield of anti-(HMXOR) IgG (mg) from third circulation**	**Total yield (mg) of anti-(HMXOR) IgG obtained from IgG prep**
256	4.8	2.1	0.7	7.6
257	3.5	1.4	0.3	5.2

Table 6.3.2 Yield of anti-(HMXOR) IgG purified from IgG by chromatography on HMXOR-affinity column

The yield of anti-(HMXOR) IgG purified from the same preparation of IgG was assessed by measuring the absorbance of the eluant at 280 nm. A_{280} (1 mg/ml) was assumed to be equivalent to an absorbance of 1.4.

6.3.4 Characterisation of affinity-purified rabbit anti-(HMXOR) IgG

The dialysed anti-(HMXOR) IgG preparation was subjected to SDS-PAGE analysis.

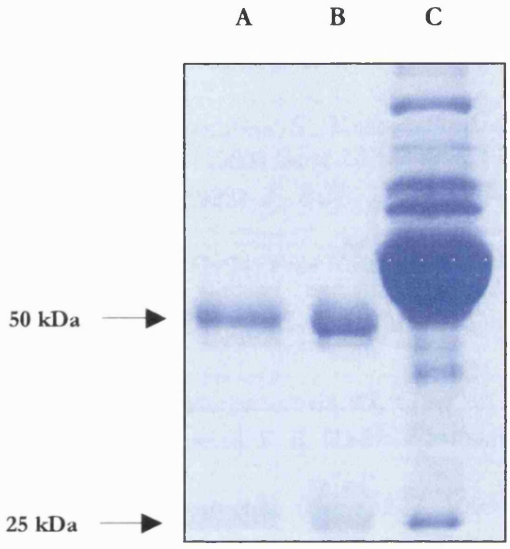


Figure 6.3.4 10 % SDS-PAGE gel of purified anti-(HMXOR) IgG

The affinity purified anti-(HMXOR) IgG was run on SDS-PAGE gel together with whole serum. Lanes A and B correspond to affinity purified anti-(HMXOR) IgG, and lane C corresponds to whole antiserum. Two bands are evident in the purified preparation, of approximately 50 kDa and 25 kDa, corresponding to heavy chain and light chain IgG respectively.

6.3.5 Specificity of purified anti-(HMXOR) IgG assessed by Western blotting

6.3.5.1 Specificity for HMXOR and BMXOR

The specificity of purified anti-(HMXOR) IgG was assessed by immunoblotting against HMXOR and BMXOR.

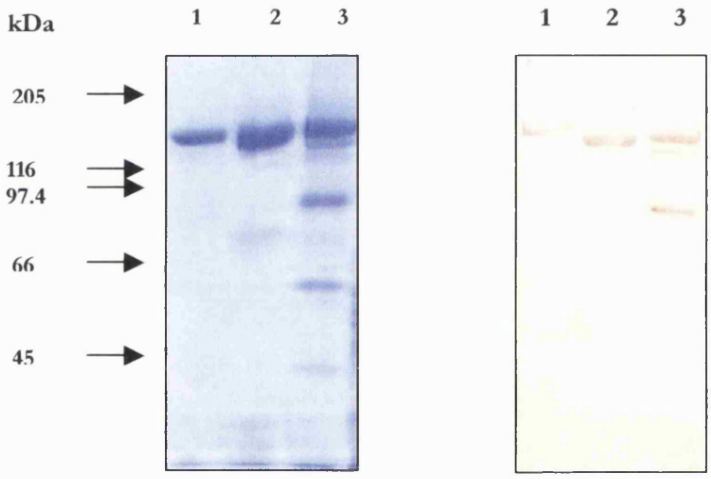


Figure 6.3.5 Western blot to show specificity of purified anti-(HMXOR) IgG for HMXOR and BMXOR

Immunoprobing was performed using the purified and pooled antibodies to test their specificity towards HMXOR and BMXOR. On both the SDS-PAGE gel and the corresponding Western blot, lane 1 corresponds to HMXOR, lane 2 corresponds to BMXOR, and lane 3 corresponds to commercial BMXOR (Biozyme). The position of

high range molecular weight markers are also shown (to the left of the SDS-PAGE gel). The Western blot demonstrates recognition of the major 150 kDa band by the purified antibodies in all cases. Commercial BMXOR (Biozyme) is a proteolysed preparation, displaying five major bands on SDS-PAGE gel. The purified antibodies appear positive for the 150 kDa, 130 kDa, and 90 kDa fragments, but do not recognise the lower molecular weight degradation products evident on the SDS-PAGE gel.

6.3.5.2 Specificity for BLXOR

The specificity of purified anti-(HMXOR) IgG was assessed by immunoblotting against bovine liver ammonium sulphate fraction, prepared as described in Section 5.2.2.1.

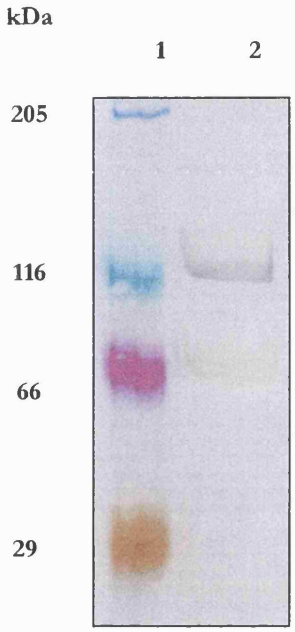


Figure 6.3.6 Western blot to show specificity of purified anti-(HMXOR) IgG for BLXOR

Immunoprobing was performed using the purified pooled antibodies to test their specificity towards BLXOR. Lane 1 corresponds to coloured high range molecular weight standards, and lane 2 corresponds to the post-ammonium sulphate fraction of bovine liver.

6.4 Discussion

High titre antisera were routinely obtained from two rabbits immunised with HMXOR; titres showing increases of approximately 400-fold compared with those of normal rabbit serum. Levels of anti-(XOR) antibodies in the rabbit serum before immunisation are low but real (Table 6.3.1). Low levels of antibodies to XOR are commonly found in most mammalian species (Bruder *et al.*, 1984).

Rabbit polyclonal anti-(HMXOR) IgG was successfully purified from rabbit antisera using a combination of ammonium sulphate precipitation, protein A-Sepharose, and HMXOR-affinity chromatography. The purified antibodies exhibited two bands on SDS-PAGE gel analysis, of 50 kDa and 25 kDa. These equate to heavy and light chain IgG respectively. In Western blots, equal avidity was displayed towards the major 150 kDa band of HMXOR and BMXOR (either prepared by methods detailed in this Chapter, or obtained commercially). In addition, the degradation fragments of the more proteolysed commercial BMXOR, corresponding to 130 kDa and 90 kDa, were recognised by the antibodies although the lower molecular weight bands were not detected (Fig 6.3.5). A band corresponding to BLXOR, at approximately 150 kDa, was detected in the post-ammonium sulphate fraction of bovine liver (Fig 6.3.6). Immunoprobing performed with normal rabbit serum gave negative results (data not shown).

It is apparent that there is cross-reactivity of the polyclonal antibodies towards XOR from human or bovine milk and from bovine liver. Such antibody binding appears to

be independent of enzyme activity, as avidity is shown for the lower activity HMXOR as well as BMXOR. The primary sequences of HMXOR and BMXOR have been shown to be essentially identical to each other (Pearson, A. Ph.D. Thesis, 2001).

It was observed that the HMXOR-affinity column lost its characteristic brown colour during initial usage, a finding attributed to the loss of the flavin group of the enzyme in response to the eluant used, 35 mM diethylamine. This did not appear to affect the performance of the column in the initial stages, indicating that the immobilised HMXOR remained antigenic. The use of alternative elution buffers in the purification of anti-(HMXOR) IgG, such as urea and magnesium chloride, has been investigated in the past by previous members of the laboratory without much success.

The performance of the column declined after several runs, as judged by a decrease in yield of antibody purified. This may be due to slow leakage of the immobilised antigen through repeated elution. However, no evidence of bands corresponding to HMXOR was seen in the eluted protein on SDS-PAGE analysis. Ligands coupled to CNBr-activated matrix have been reported to show a greater degree of leakage than those coupled using other methods (Harris & Angal, 1995), perhaps because the covalent bonds between ligand and matrix are relatively labile. Nevertheless, for the purpose of antibody purification, this did not appear to be too problematic as the combined yields produced from repeated purifications, until the column significantly lost performance, were sufficient for considerable experimentation. Certainly, sufficient antibody was

available for coupling to a matrix to form an immunoaffinity column, preparation and use of which are discussed in the next Chapter.

7. The immunoaffinity purification of bovine liver xanthine oxidoreductase (BLXOR)

7.1 Introduction

Immunopurification can overcome separation difficulties that other methods are unable to resolve, as antibodies can be found that are able to distinguish between very similar antigens (Jack *et al.*, 1987). Antibodies are particularly resistant to proteolytic attack by enzymes and thus are able to offer a clear advantage over affinity methods utilising immobilised proteins as ligands. Immunopurification is, accordingly, an attractive option for the purification of any protein against which an antibody, polyclonal or monoclonal, can be raised.

Affinity chromatography has been rarely used early on in a purification procedure (Bonnerjea *et al.*, 1986), where the potentially high degree of purification would be most useful. This is probably because of the risk of damage to a valuable material, both from fouling by high levels of contaminants and proteolysis. Antibodies, whether polyclonal or monoclonal, are often produced by time-consuming and labour intensive methods.

There have been no reported immunopurifications of XOR from bovine liver, although a purification has been reported from human liver (Saksela *et al.*, 1999). This Chapter investigates the use of an immunoaffinity matrix, prepared using affinity-purified

polyclonal anti-(HMXOR) antibodies, as prepared in the previous Chapter, in the purification of XOR from bovine liver.

7.2 Materials and Methods

7.2.1 Materials

All chemicals were obtained from Sigma-Aldrich, Poole, Dorset, unless stated otherwise.

7.2.2 Methods

7.2.2.1 Preparation of a polyclonal anti-(HMXOR) IgG column

As already noted, Protein A has a high specificity for the Fc domain of most classes of rabbit IgG. Coupling of antibodies directly to Protein A beads via their Fc domain, leaves the antigen-binding site free and available for interaction.

Anti-(HMXOR) antibodies (Section 6.2.2.6) were incubated (4 mg/ml) with wet Protein A-Sepharose beads (swollen in PBS). The antibody and Protein A-Sepharose slurry was incubated at room temperature for 1 h, with gentle rocking on a shaker. The beads were washed twice with ten volumes of 0.2 M Na-borate, pH 9. Washes were performed by centrifugation at 3000 g for 5 min, and aspiration of the resulting supernatant. The beads were resuspended in ten volumes of 0.2 M Na-borate, pH 9, and dimethylpimelimidate (a bifunctional coupling reagent) was added to bring the final concentration to 20 mM. The suspension was mixed on a shaker at room temperature for 30 min and the reaction was stopped by washing with 0.2 M ethanolamine, pH 8, and incubation at room temperature for 2 h with gentle mixing. This ensured blocking

of uncoupled sites. The beads were resuspended in PBS (containing 0.01 % ($^{w}/_{v}$) sodium azide), poured into a column and stored at 4 °C until ready for use.

Coupling was assessed by taking small samples of the beads before and after coupling (with dimethylpimelimidate) and analysing using SDS-PAGE to check for presence of antibody. Efficient coupling is indicated by the absence of bands corresponding to antibody heavy chain in the sample taken after coupling.

7.2.2.2 Preparation of ammonium sulphate fraction from liver

An ammonium sulphate fraction was prepared from bovine liver and dialysed against PBS as described in Section 5.2.2.1. Protease inhibitors were added to the liver fraction as follows: 5 μM leupeptin, 10 μM chymostatin, 1 μM pepstatin A and 10 μM APMSF. The inclusion of protease inhibitors is crucial at this stage, both to minimise proteolysis of protein and also to protect the valuable antibody matrix used at the next stage, from degradation.

7.2.2.3 Pre-purification of liver ammonium sulphate fraction on Protein A-Sepharose

Protein A-Sepharose (4 ml) was poured into a column and equilibrated in PBS. The ammonium sulphate liver fraction, dialysed against PBS (Section 7.2.2.2), was circulated for 2 h at 4 °C on an uncoupled Protein A-Sepharose column, pre-equilibrated in PBS. This was to minimise non-specific binding of protein to the matrix and also to remove any IgG that may be present in the liver fraction and potentially co-purify with XOR. Bound protein was eluted with 35 mM diethylamine, containing 35 mM NaCl, pH 10.5,

and discarded. The non-bound protein fraction was retained for immunoaffinity purification.

7.2.2.4 Immunoaffinity purification of BLXOR

The ammonium sulphate fraction of bovine liver, which had been pre-treated with Protein A-Sepharose (Section 7.2.2.3), was diluted 1:10 in PBS (containing 0.01 % ($^{w}/_{v}$) sodium azide) and applied to the anti-(HMXOR) immunoaffinity column (Section 7.2.2.1), which had been previously equilibrated in the aforementioned buffer. Circulation was allowed to occur for 2-3 h at 4 °C in order to saturate antibody-binding sites. Following circulation, the column was washed extensively with PBS, followed by PBS containing 1 M NaCl, to remove unbound or weakly bound protein, until A_{280} reached baseline values. To elute XOR, 25 mM diethylamine, containing 35 mM NaCl, pH 10.5, was applied to the column. The bound protein was collected in 50 mM Na-Bicine, pH 8.3 (5-fold excess) to neutralise the alkalinity of the diethylamine. The eluted fraction was immediately dialysed against one change of 50 mM Na-Bicine, containing 0.05 M NaCl, 1 mM EDTA and 4 mM DTT, pH 8.3. The dialysed material was incubated with 1 mM FAD for 10 min at room temperature.

Incubation with FAD was carried out in order replace flavin loss from XOR. Such loss has been observed upon incubation of intact HMXOR with diethylamine (personal communication, R. Bryant). FAD is rapidly hydrolysed to riboflavin 4'-5'-cyclic phosphate in alkali solutions. Therefore, the FAD incubation step has been incorporated following dialysis of the eluted protein against 50 mM Na-Bicine, pH 8.3. Following incubation with FAD, further dialysis was carried out against several changes

of 50 mM Na-Bicine, containing 0.05 M NaCl, 1 mM EDTA and 4 mM DTT, pH 8.3, to remove the unbound FAD.

The same liver fraction was subjected to four consecutive circulations and elutions from the immunoaffinity column. Following each elution of bound protein, the column was regenerated by washing with PBS, followed by 0.1 M Na-acetate, pH 4.5. When not in use, the column was stored in PBS with 0.01 % ($^{w}/_{v}$) sodium azide added to prevent microbial growth. Enzyme was concentrated using Vivaspin 2 ml concentrators (Vivascience), drip-frozen into liquid nitrogen and stored at -70°C.

7.3 Results

7.3.1 Pre-purification of liver ammonium sulphate fraction on Protein A-Sepharose

Bovine liver (40 g) was homogenised and an ammonium sulphate fraction was prepared (Section 5.2.2.1). The ammonium sulphate fraction was then circulated on an uncoupled Protein A-Sepharose column (Section 7.2.2.3), to remove protein with an affinity for Protein A, prior to immunoaffinity purification on Protein A-antibody matrix.

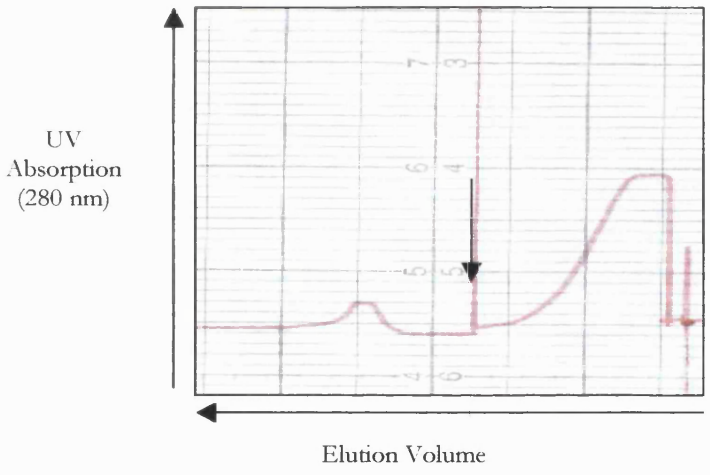


Figure 7.3.1 Elution profile from Protein A-Sepharose

There was an apparent small increase in the A_{280} upon elution with 35 mM diethylamine, containing 35 mM NaCl, pH 10.5 (highlighted by arrow). The eluted fraction was subsequently electrophoresed on SDS-PAGE gel but no protein was detected,

indicating that protein quantity was minimal. Alternatively, the small increase in absorbance could be a consequence of buffer change. The non-bound protein fraction was retained for immunoaffinity chromatography.

7.3.2 Immunoaffinity chromatography on anti-(HMXOR) column

The resulting liver protein was subjected to immunoaffinity chromatography (Section 7.2.2.4). The traces below show three typical consecutive elutions from the anti-(HMXOR) immunoaffinity column, monitoring absorbance at 280 nm.

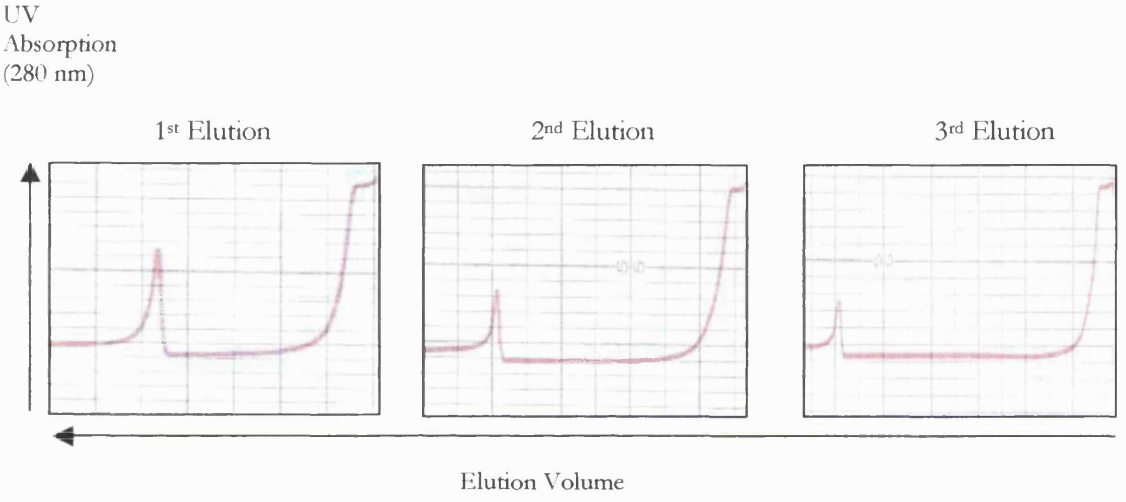


Figure 7.3.2 Elution profiles showing elution of bound protein from anti-(HMXOR) immunoaffinity column

In all three consecutive circulations bound protein eluted from the column with 25 mM diethylamine, containing 35 mM NaCl, pH 10.5. This is represented by the sharp peak on the left of each trace. This peak gets smaller with progressive circulations on the column, indicating removal of XOR protein from the liver fraction.

7.3.3 SDS-PAGE gel of immunoaffinity-purified protein

Following incubation with 1 mM FAD and further dialysis (Section 7.2.2.4), the purified protein was subjected to SDS-PAGE analysis.

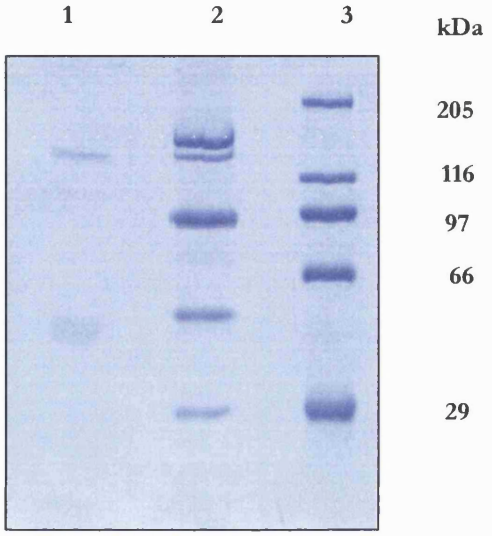


Figure 7.3.3 7.5 % SDS-PAGE gel of immunoaffinity-purified protein

Lane 1 corresponds to immunoaffinity-purified protein, lane 2 corresponds to commercial BMXOR (Biozyme) and lane 3 corresponds to high range molecular weight standards. It can be seen that the purified BLXOR runs with a mobility equal to that of the second highest molecular weight band of commercial BMXOR. It can also be observed that there is an additional faint lower molecular weight band.

7.3.4 Western blot of immunoaffinity-purified protein

The purified protein was subjected to Western blotting (Section 3.2.1.3), whereby monoclonal mouse anti-(HMXOR) (clone 1D9D1), kindly supplied by Eurogenetics, Belgium, was used as primary antibody.

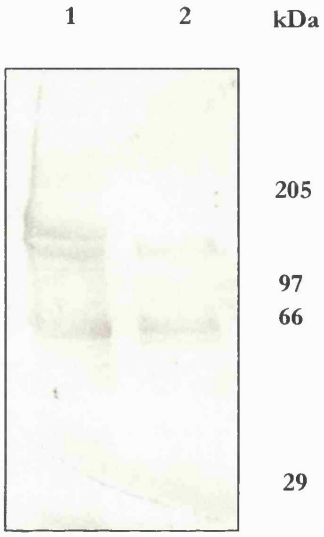


Figure 7.3.4 Western blot of immunoaffinity-purified BLXOR

Lane 1 corresponds to commercial BMXOR and lane 2 corresponds to immunoaffinity-purified protein. As in the SDS-PAGE gel (Fig 7.3.3), it can be seen that the immunopurified protein runs as two bands, both bands of similar molecular weight as those represented in lane 1, corresponding to commercial BMXOR.

7.3.5 Molecular weight

The molecular weights of immunoaffinity-purified BLXOR and commercial BMXOR as estimated by SDS-PAGE gel (Fig 7.3.3) were determined by plotting log molecular weight against R_f.

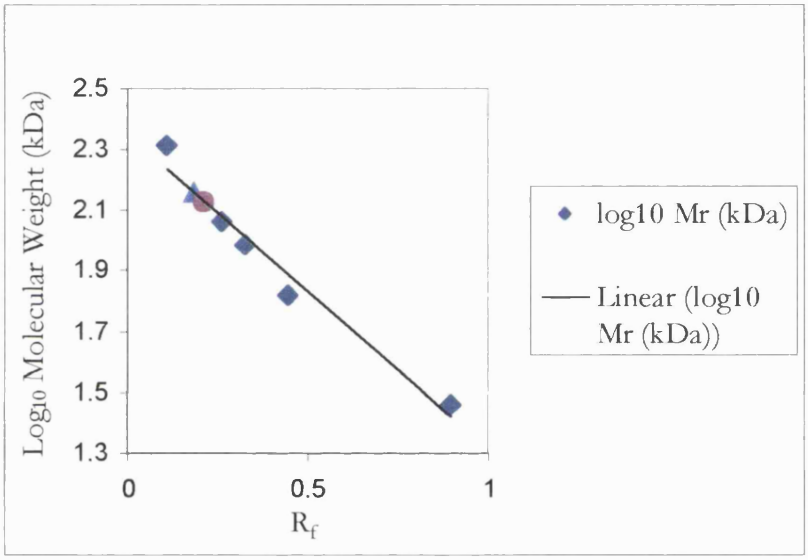


Figure 7.3.5 Determination of the molecular weight of BLXOR and BMXOR by SDS-PAGE gel

Molecular weight was determined as described in Section 3.2.1.1. Molecular weight standards are represented by squares (blue), BMXOR is represented by triangle (light blue) and BLXOR by circle (red). The calculated molecular weights of BMXOR (major band) and BLXOR are 145 kDa and 134 kDa respectively.

7.3.6 UV-visible absorbance spectrum of immunoaffinity-purified protein

The UV-visible absorbance spectrum of the immunoaffinity-purified protein is shown below.

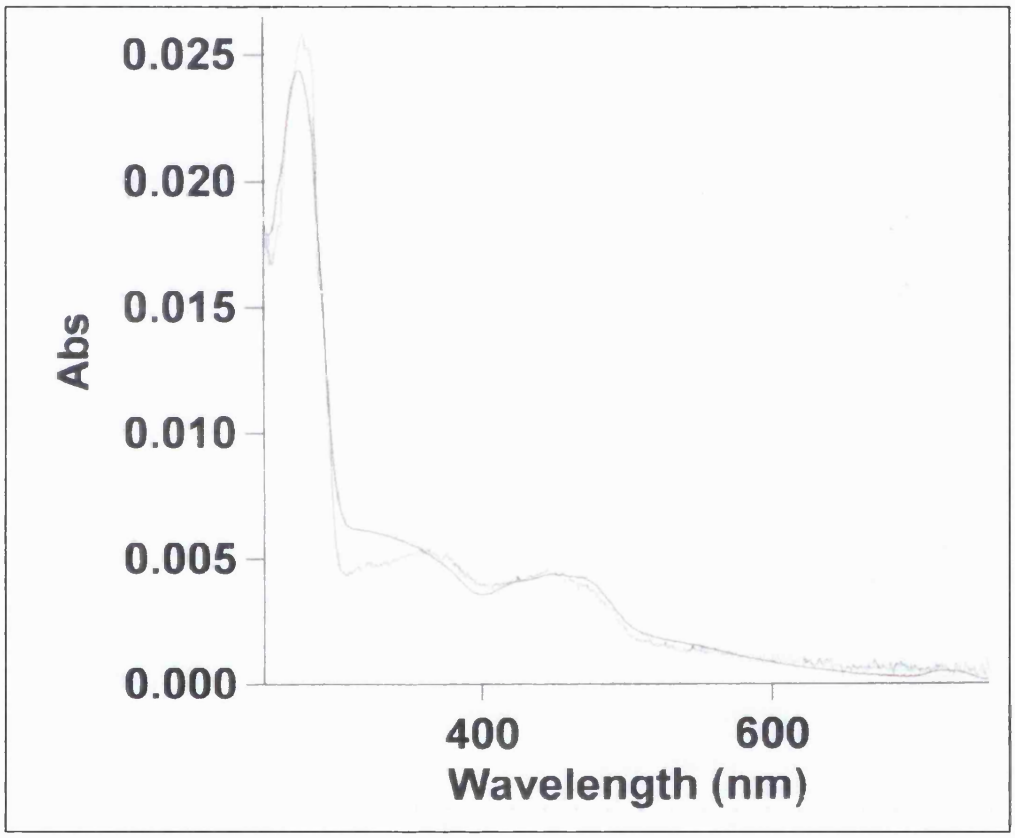


Figure 7.3.6 UV-visible absorbance spectra of immunoaffinity-purified BLXOR and purified HMXOR

BLXOR is represented by the grey line and HMXOR is represented by the red line. The spectra were obtained in 50 mM Na-Bicine, containing 0.05 M NaCl, pH 8.3.

7.3.7 Specific activity of immunoaffinity-purified protein

The specific activities were calculated at each stage of the purification procedure. Typical results are shown below.

Purification Step	Total Activity (nmol/min)	Total Protein (mg)	Specific Activity (nmol/min/mg)	% Oxidase
Ammonium sulphate fraction	0.09	53	5	24
Immunoaffinity-purified fraction	936	1.3	720	46

Table 7.3.1

Urate activity assays (Section 3.2.4.2) were performed on the ammonium sulphate fraction from bovine liver prior to immunoaffinity column purification, and on the purified protein following immunoaffinity column chromatography. The total yield of immunoaffinity-purified protein was 1.3 mg from 40 g liver.

7.4 Discussion

With the use of a three-step purification method, consisting of ammonium sulphate precipitation, circulation on Protein A-Sepharose and a single immunoaffinity step, XOR has been purified from bovine liver to near homogeneity as judged by SDS-PAGE gel. Furthermore, this purified preparation exhibited a UV-visible spectrum bearing strong similarities to that of purified human milk XOR.

The post ammonium sulphate bovine liver fraction was circulated on uncoupled Protein A-Sepharose (Fig 7.3.1), in order to remove any IgG likely to be present in liver homogenate. Other proteins with affinity for Protein A itself, as apposed to Protein A coupled to anti-(HMXOR) antibody, will also be removed prior to immunoaffinity purification on the latter column. Immunoaffinity chromatography, involving three consecutive circulations and elutions on the anti-(HMXOR) column, resulted in a distinct peak at 280 nm (Fig 7.3.2), corresponding to the elution of bound protein from the column in each case. There is a decrease in size of peak noted with progressive elutions, indicating removal and reduction of XOR protein in the liver fraction. Complete removal of XOR from the liver fraction was routinely observed after three elutions, as a further circulation and elution produced no change in absorbance at 280 nm.

SDS-PAGE analysis of purified protein (Fig 7.3.3) showed a band of approximately 134 kDa, calculated by plotting log molecular weight against R_f (Fig 7.3.5), and a lower molecular weight band of approximately 50 kDa. Western blotting performed using a

monoclonal anti-(HMXOR) antibody (Fig 7.3.4), and therefore a different antibody to the polyclonal used in purification, was specific towards both the 134 kDa band and the 50 kDa band. This indicates that the band corresponding to 134 kDa is XOR and the lower molecular weight band is likely to be an XOR proteolysis product. The 134 kDa band is similarly sized to the second largest proteolysis or degradation product of commercial BMXOR, as can be seen from both the SDS-PAGE gel and Western blot. The 50 kDa band, initially thought to be a contaminant, is recognised by the monoclonal antibody and is also of a similar size to a proteolysis fragment of commercial BMXOR. It can thus be assumed that the purified BLXOR is mildly proteolysed, although to a lesser extent than commercial BMXOR, with BLXOR producing only two visible bands on SDS-PAGE gel compared with five distinct bands belonging to commercial BMXOR. Whether the 135 kDa band represents a proteolysis product as well, equivalent to those bands of similar molecular weight observed with BMXOR and HMXOR, is unclear. There is no evidence of a higher molecular weight band representing unproteolysed enzyme. It is interesting to note that the two XORs, when subjected to proteolysis, produce similarly sized fragments. Proteolysis of rat liver XDH with trypsin cleaves the enzyme into three fragments of 20 kDa, 40 kDa, and 85 kDa (Amaya *et al.*, 1990). This cleavage is accompanied by the conversion of the dehydrogenase to the oxidase form of the enzyme (Hille & Nishino, 1995). However, this represents considerably more fragmentation than that observed in the present work.

The molecular weight of BLXOR was estimated (Section 7.3.5), by plotting log molecular weight against R_f calculated from SDS-PAGE, and was found to be 134 kDa.

The major band of commercial BMXOR was calculated to be 145 kDa. This method of measuring molecular weight shows a slightly lower value for BLXOR than for BMXOR. The molecular weight of 134 kDa compares well with the BLXOR preparation of Cabre & Canela (1986), which upon SDS-PAGE analysis showed four protein bands, two of which possessed molecular masses of 135 kDa and 45 kDa, the latter also being a similar mass to the lower band obtained here. In the present work it is possible that the band at 135 kDa, in addition to the band at 50 kDa, represents a proteolysis product. However, BLXOR purified using non-immune methods (Chapter 5) showed a very close molecular weight of 135 kDa, and a similarly sized band to this was also noted in freshly prepared homogenate on Western blotting (Fig 5.3.4), indicating that perhaps this is not due to proteolysis, and instead may indicate a smaller size XOR, when compared with BMXOR and HMXOR.

The epitope sequence recognised by the monoclonal antibody used in Western blotting is again clearly conserved between bovine liver and bovine milk XORs (as discussed previously in Section 6.4), accounting for the binding of monoclonal antibody to XOR degradation products. From observations so far, it also appears that the affinity-purified polyclonal anti-(HMXOR) antibody and the monoclonal anti-(HMXOR) antibody (Eurogenetics, Belgium) have similar specificities.

The yield of immunopurified BLXOR was 1.3 mg, from 40 g liver tissue. This is over 10-fold higher than the yield obtained using the affinity purification methods (0.1 mg) in Chapter 5, using a similar starting amount of liver (Section 5.3.6). The specific activity

of the immunopurified preparation (720 nmol/min/mg) is also significantly higher than the activity reported for BLXOR purified using non-immune methods (1.6 nmol/min/mg), described in the previous Chapter (Table 5.3.2).

Purified BLXOR displayed a UV-visible absorption spectrum very similar to that of HMXOR, which also resembles BMXOR (Fig 7.3.6). Peaks were present at approximately 320 and 450 nm; absorbance in this region is contributed by the flavin and Fe/S groups. However, there is a lower absorbance in the 320-350 nm region of BLXOR, when compared with the spectrum of HMXOR. This may be due to a lower composition of either flavin or iron-sulphur in the purified enzyme, upon comparison with HMXOR. If the preparation of BLXOR is lacking in one or both of these groups, this, as discussed in the Introduction, will result in a lowered activity enzyme, depending on the extent of this deficiency. It is possible that the formation of deflavo-XOR during this purification will occur, due to the use of diethylamine, which, as an elution buffer, has been found to strip enzyme of its FAD group. Deflavo-XOR can also be prepared within the laboratory by incubation with a range of bivalent metal salts, such as calcium chloride and magnesium sulphate (Morell, 1952). Komai *et al.* (1969) found that high concentrations of calcium chloride result in the hydrolysis of FAD to FMN, producing an altered UV-visible spectrum. This group further demonstrated that deflavo-XOR could be reconstituted with the addition of FAD, restoring the majority (60 %) of the original enzymic activity. To correct for loss of flavin in this study, addition of FAD to the preparation following elution from the column and subsequent dialysis has been incorporated. The PFR calculated from this UV-visible scan is 5.75,

indicative of reasonably pure enzyme. Highly purified HMXOR and BMXOR within the laboratory typically have PFRs in the range of 5.2 – 5.7.

The BLXOR produced by the methods described in this Chapter consists of 46 % oxidase form (Table 7.3.1). The proportion of oxidase enzyme has thus increased from 24 % to 46 % during immunoaffinity purification. DTT (4 mM) has been incorporated at each stage in the procedure to prevent spontaneous formation of reversible XO and to retain the enzyme in its native dehydrogenase form. The fact that we see an increase in oxidase form following immunoaffinity purification may be due to proteolysis or protein degradation.

It appears from these results that immunoaffinity chromatography utilising polyclonal antibodies is an extremely successful tool in the isolation of BLXOR from a heterogeneous protein mixture, without proving too deleterious to the activity and properties of the enzyme, as shown by activity assays and UV-visible scan. The polyclonal antibodies utilised demonstrate a high specificity towards BLXOR and are relatively inexpensive to incorporate as a ligand in an immunoaffinity column, as several purifications can be performed until the column shows a loss in performance.

8. The immunoaffinity purification of human liver xanthine oxidoreductase (HLXOR)

8.1 Introduction

XOR has been purified from human liver (Krenitsky *et al.*, 1986; Moriwaki *et al.*, 1993; Saksela *et al.*, 1999). In 1986, Krenitsky *et al.* purified XOR 2000-fold from post-mortem human liver, using a combination of affinity procedures. Their preparation was moderately proteolysed and in its oxidase form, displaying a specific activity, with purine substrates, similar to that of bovine milk XOR. This is in contrast to human milk XOR, which has a much lower specific activity. Parks & Granger (1986) found XOR activity in whole human tissues to be generally low, with the exception of liver and intestine. While this could reflect a higher content of the enzyme in the liver and intestine, it would also be consistent with a relatively high specific activity of XOR in these two tissues.

In 1993, Moriwaki *et al.* reported the affinity purification of XOR from cadaver liver. They obtained a final purification of 1600-fold, claiming homogeneity as judged by SDS-PAGE, with an estimated mass of 150 kDa, although these data are not shown. They went on to use the purified enzyme to raise polyclonal antibody in rabbits, for tissue immunolocalisation studies. That their enzyme is apparently unproteolysed differs from the earlier purification of liver enzyme (Krenitsky *et al.*, 1986), SDS-PAGE of which showed five bands. Krenitsky *et al.* prepared XOR from human liver total

homogenate, whereas Moriwaki *et al.* used human liver cytosol as a basis for their purification. This may have affected the differing extent of proteolysis. Alternatively, proteolysis may have occurred to a greater extent in the post-mortem tissue, used by Krenitsky *et al.*, prior to purification.

Sarnesto *et al.* (1996), used fresh human liver, preserved for transplantation, then frozen in liquid nitrogen, before homogenisation in the presence of protease inhibitors. The homogenate itself showed multiple bands on Western blotting with polyclonal anti-(HMXOR) antibody, leading them to suggest that the enzyme might exist in a partially proteolysed form in the liver, and possibly other intact tissue.

Saksela *et al.* (1999) reported the immunoaffinity purification of XOR from human liver, using rabbit polyclonal anti-(XOR) antiserum coupled to Sepharose. The purified liver protein showed three bands on SDS-PAGE analysis, of 150 kDa, 130 kDa and 85 kDa. Their method of purification was not described in detail, however, nor were the SDS-PAGE results shown in the report. This study also proposed that cytoplasmic human liver XDH is irreversibly cleaved to XO by a heat-labile mitochondrial protease, which is released from the mitochondrial intermembrane space into the cytosol upon mitochondrial damage. This would support the claims of Moriwaki *et al.* (1993) to have purified unproteolysed XOR from liver cytosol.

Hellsten-Westing (1993) generated a range of monoclonal antibodies in order to determine the tissue distribution of the enzyme. As part of the study they performed

affinity chromatography on human liver and skeletal muscle extracts, using monoclonal antibody immobilised to Protein A-Sepharose. They found that the eluate from human liver contained a fragmented enzyme as judged by SDS-PAGE, with molecular weights of 155 kDa, 143 kDa and 95 kDa. Their monoclonal antibodies also bound a 155 kDa protein displaying xanthine oxidase activity in human milk and skeletal muscle, reflecting tissue cross-specificity of their antibody.

The work in this Chapter extends to human liver the immunoaffinity methodology developed for the isolation of XOR from bovine liver tissue (Chapter 7). The efficacies of polyclonal and monoclonal antibodies as purification ligands are compared.

8.2 Materials and Methods

8.2.1 Materials

Frozen human liver (normal and primary biliary cirrhotic) was obtained from the Royal Free Hospital, London. Sephadex G-25 PD-10 gel filtration columns were obtained from Pharmacia, Uppsala, Sweden. CNBr-activated Sepharose 4B was supplied by Amersham Pharmacia Biotech AB. All other chemicals were obtained from Sigma-Aldrich, Poole, Dorset, unless stated otherwise. Monoclonal anti-(HMXOR) antibody clones (1D9D1, 3E7A6, 3C9D5) were kindly donated by Eurogenetics, Belgium. Protein L-Agarose was obtained from Sigma–Aldrich.

8.2.2 Methods

8.2.2.1 Homogenisation of human liver and preparation of an ammonium sulphate fraction

All operations were carried out in a fume cupboard and all equipment was cooled to 4 °C before use. Suitable precautions were taken when handling human tissue and to avoid contamination of apparatus. Frozen human liver (40 g) was thawed on ice, and rinsed in ice-cold PBS, containing protease inhibitors as follows: 5 μM leupeptin, 10 μM chymostatin, 1 μM pepstatin A and 10 μM APMSF, with the addition of 1 mM EDTA and 4 mM DTT. Homogenisation, followed by ammonium sulphate precipitation, was carried out as described in Section 5.2.2.1. The resultant pellet from ammonium sulphate precipitation was redissolved in PBS. Dialysis of the ammonium sulphate

suspension was carried out overnight against two changes of PBS (3000 ml), containing protease inhibitors as previously described, together with 1 mM EDTA and 4 mM DTT. The dialysed ammonium sulphate liver fraction was syringe-filtered using a 0.2 μm disposable filter. Prior to further purification, low molecular weight inhibitors, such as purines, were removed by gel filtration using a Sephadex G-25 PD-10 column (Section 3.2.2).

For requirements of small amounts of crude liver protein (e.g. for activity and protein assays, or SDS-PAGE and Western blotting purposes), the ammonium sulphate liver fraction was frozen. The pelleted fraction from ammonium sulphate precipitation was redissolved in 50 mM Na-Bicine, containing 1 mM EDTA and 4 mM DTT, pH 8.3, and dialysed against the same buffer. Phosphate buffer was avoided in this case, as the pH drop upon freezing in this buffer is likely to be detrimental to the protein. Following dialysis, the crude liver protein was drip-frozen into liquid nitrogen and stored at –70 °C for future use. Rapid freezing in this way did not appear to influence the specific activity of the enzyme, nor did it seem to initiate dehydrogenase to oxidase conversion.

8.2.2.2 Pre-purification of liver ammonium sulphate fraction

The ammonium sulphate liver fraction, dialysed against PBS (Section 8.2.2.1), was circulated for 2 h at 4 °C on an uncoupled Protein A-Sepharose column, pre-equilibrated in PBS (Section 7.2.2.3).

8.2.2.3 Preparation of a polyclonal anti-(HMXOR) column

A polyclonal anti-(HMXOR) immunoaffinity column (3-4 ml) was prepared as described in Section 7.2.2.1.

8.2.2.4 Preparation of a monoclonal anti-(HMXOR) column

Two ways of immobilising monoclonal antibody using different means of coupling were investigated, coupling to CNBr-activated Sepharose and coupling to Protein A-Sepharose. Mouse monoclonal anti-(HMXOR) clone 1D9D1 was selected for immunoaffinity column use because it was found to be highly specific, as can be seen from the following Results section.

8.2.2.4.1 Coupling to CNBr-activated Sepharose 4B

The antibody solution (10 mg) was extensively dialysed against 0.5 M Na-phosphate, pH 7.5, to remove extraneous compounds containing amino or thiol groups (which would interfere with the coupling). The CNBr-activated Sepharose 4B beads (1 ml) were added (after which a small volume was routinely retained and assayed in order to determine the coupling efficiency), and the suspension was mixed gently at room temperature overnight on a rocker. The beads were washed twice with 0.5 M Na-phosphate, pH 7.5, and then with 0.05 M Na-phosphate, containing 2 M NaCl, pH 7.5. 10 vol of 100 mM ethanolamine, pH 7.5, were then added, followed by incubation at room temperature overnight with gentle rocking. The coupling efficiency was calculated at this stage and was consistently between 70 % and 80 %. Two washes were

performed with PBS, and 0.01 % ($^{w}/_{v}$) sodium azide was added. The beads were poured into a column and the column matrix was stored at 4 °C until required.

8.2.2.4.2 Coupling to Protein A-Sepharose

Protein A has varying affinities for antibodies from different species, classes and subclasses. Although it has a high affinity for rabbit IgG (all subclasses), it has a much lower affinity for mouse IgG, particularly mouse IgG_1. The mouse monoclonal 1D9D1 antibody used for the present studies was of the subclass IgG_1, necessitating coupling under conditions modified from those used when preparing the polyclonal rabbit anti-(HMXOR) IgG column (as described in Section 7.2.2.1).

The affinity of IgG_1 for Protein A can be increased by adjusting the binding conditions by using high salt concentrations that favour the hydrophobic Protein A-Fc binding. The antibody solution 1D9D1 (10 mg at 5 mg/ml) was adjusted to pH 9, and NaCl was added to raise the concentration to 3 M. The antibody was mixed with Protein A beads (2 ml) at room temperature for 1 h, with gentle rocking. The beads were washed twice with 10 volumes of 50 mM Na-borate, containing 3 M NaCl, pH 9, by centrifugation and aspiration. The beads were resuspended in 10 volumes of 0.2 M Na-borate, containing 3 M NaCl, pH 9. Dimethylpimelimidate (solid) was added to bring the final concentration to 20 mM. The suspension was mixed at room temperature for 30 min on a shaker. The reaction was stopped by washing the beads in 0.2 M ethanolamine, pH 8, incubating at room temperature for 2 h with gentle mixing. The beads were

resuspended in PBS with 0.01 % ($^{w}/_{v}$) sodium azide, and poured into a column. The column matrix was stored at 4 °C until required.

8.2.2.5 Polyclonal immunoaffinity purification of HLXOR

This method is the same as that described in the previous Chapter (Section 7.2.2.4) for the purification of XOR from bovine liver. The ammonium sulphate fraction of human liver, which had been pretreated with Protein A-Sepharose (Section 8.2.2.2), was diluted 1:10 in PBS (containing 0.01 % ($^{w}/_{v}$) sodium azide) and applied to the anti-(HMXOR) immunoaffinity column (Section 8.2.2.3), which had been previously equilibrated in the aforementioned buffer. Circulation was allowed to occur for 2-3 h at 4 °C in order to saturate antibody-binding sites. Following circulation, the column was washed extensively with PBS, followed by PBS containing 1 M NaCl, to remove unbound or weakly bound protein, until A_{280} reached baseline values. To elute XOR, 25 mM diethylamine, containing 35 mM NaCl, pH 10.5, was applied to the column. The bound protein was collected in 50 mM Na-Bicine, pH 8.3 (5-fold excess), to neutralise the alkalinity of the diethylamine. The eluted fraction was immediately dialysed against one change of 50 mM Na-Bicine, containing 0.05 M NaCl, 1 mM EDTA and 4 mM DTT, pH 8.3. The dialysed material was incubated with 1 mM FAD for 10 min at room temperature. Following incubation with FAD, further dialysis was carried out against several changes of 50 mM Na-Bicine, containing 0.05 M NaCl, 1 mM EDTA and 4 mM DTT, pH 8.3, to remove the excess FAD.

The same liver fraction was subjected to four consecutive circulations and elutions from the immunoaffinity column. Following each elution of bound protein, the column was regenerated by washing with PBS, followed by 0.1 M Na-acetate, pH 4.5. When not in use the column was stored in PBS with 0.01 % ($^{w}/_{v}$) sodium azide added to prevent microbial growth.

8.2.2.6 Immunoprecipitation

The antibody solution 1D9D1 (2 mg, 3 mg/ml) was coupled to CNBr-activated Sepharose beads (200 µl) (Section 8.2.2.4.1). The beads were incubated with 200 µl ammonium sulphate human liver fraction (prepared as described in Section 8.2.2.1) for 2 h at 4 °C with gentle rocking. The beads were washed three times with PBS by centrifugation and aspiration. 6 M urea was added to dissociate bound protein from the beads, followed by centrifugation. The supernatant was electrophoresed (Section 3.2.1.1).

8.2.2.7 Monoclonal immunoaffinity purification of HLXOR

This was performed essentially as described in Section 8.2.2.5, except that 100 mM glycine, pH 2.5, replaced 25 mM diethylamine, containing 35 mM NaCl, pH 10.5, as an eluant.

8.2.2.8 Removal of immunoglobulin by circulation on Protein L-Sepharose

This step was performed immediately after immunoaffinity chromatography to remove immunoglobulin that may have co-purified with XOR, or have leached from the immunoaffinity column. Protein L is produced by *Peptostreptococcus magnus* and contains four immunoglobulin-binding domains. Unlike other bacterial immunoglobulin (Ig) binding proteins such as Protein A and Protein G, Protein L binds Ig through the κ light chain. As a result, Protein L binds with equal strength to all subclasses of human, mouse and rat IgG. In addition, it binds strongly to IgA, IgD, IgE, and IgM, as well as to Fab and scFv fragments.

Protein L-Sepharose (4 ml) was poured into a column and equilibrated in 50 mM Na-Bicine, pH 8.3. The immunopurified liver fraction (Sections 8.2.2.5 and 8.2.2.7) was filtered and circulated on an uncoupled Protein L-Sepharose column for 2 h at 4 °C in order to remove immunoglobulin. Such a procedure is termed 'negative' or subtractive immunopurification. Bound protein was eluted with 0.1 M glycine, pH 2. The bound fraction was discarded, and the remaining circulated fraction was retained. Enzyme was concentrated using Vivaspin 2ml concentrators (Vivascience), drip frozen into liquid nitrogen and stored at -70°C.

8.2.2.9 Blotting method for N-terminal protein sequencing

A 7.5 % reducing SDS-PAGE gel was run as described in Section 3.2.1.1. Powder-free, low protein gloves were worn at all times. The blotting buffer contained 10 mM CAPS

(4.42 g), 10% MeOH (200 ml) made up to 2000 ml, pH 11. Membrane (Problot) was cut to the size of the gel, and soaked in methanol for a few minutes. Two pieces of 3 mm filter paper and fibre pads were also cut to the same size as the gel and soaked in blotting buffer. The gel was soaked in blotting buffer for at least 10 min, with frequent changes, to remove all traces of glycine. Blotting was carried out essentially as described in Section 3.2.1.2. The transfer was run at 300 mA for 2 h, ensuring that the voltage did not go over 100 V. Following transfer, membrane was stained with 0.1 % Amido Black (40 % methanol/1 % acetic acid) for 30 s. Destaining was carried out by agitation of membrane in distilled water, with frequent changes. Bands of interest were cut from the membrane and stored in an Eppendorf tube at –20 °C until ready for sequencing.

8.3 Results

8.3.1 Polyclonal immunoaffinity purification of HLXOR

Ammonium sulphate fraction from human liver was first circulated on an uncoupled Protein A-Sepharose column (Section 8.2.2.2), and then subjected to immunoaffinity chromatography (Section 8.2.2.5).

8.3.1.1 SDS-PAGE gel and Western blot

Upon elution from the anti-(HMXOR) immunoaffinity column, with 25 mM diethylamine, containing 35 mM NaCl, pH 10.5, a peak was detected by UV absorbance at 280 nm. The protein representing this peak was collected, dialysed, incubated with 1 mM FAD, and further dialysed (Section 8.2.2.5). Following dialysis, the eluted fraction was concentrated using Vivaspin concentrators (Vivascience). The SDS-PAGE results are shown below.

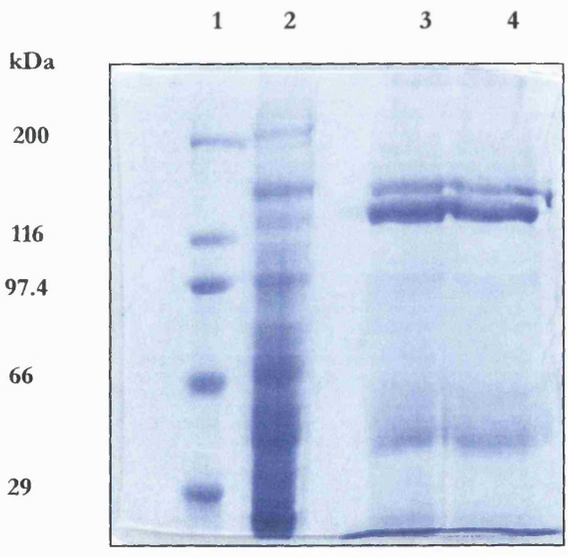


Figure 8.3.1 7.5 % SDS-PAGE gel of immunoaffinity-purified protein

Lane 1 corresponds to high range molecular weight standards, lane 2 corresponds to human liver homogenate and lanes 3 and 4 correspond to immunoaffinity-purified protein (15 μg).

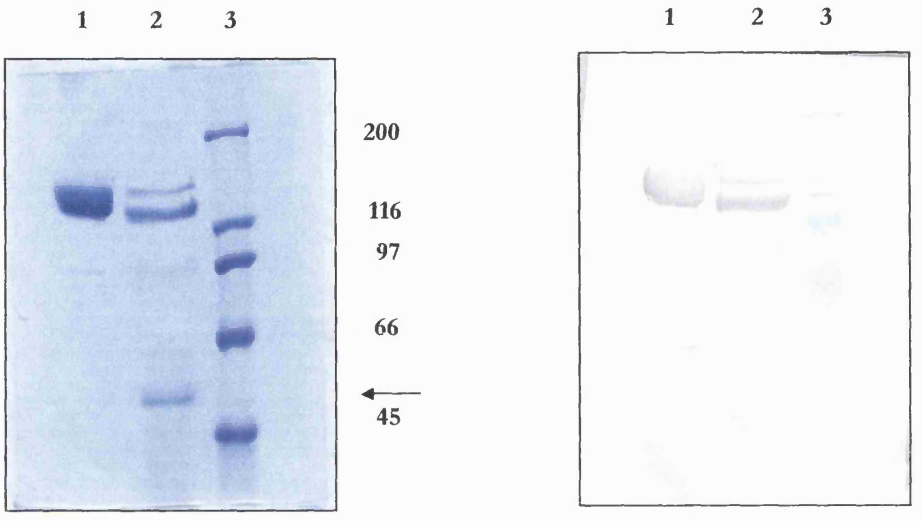


Figure 8.3.2 7.5% SDS-PAGE gel and Western blot

In both the SDS-PAGE gel and corresponding Western blot, lane 1 corresponds to HMXOR, lane 2 corresponds to immunoaffinity-purified protein (15 μg) and lane 3 corresponds to high range molecular weight standards, with coloured standards used for the Western blot. Western blotting (Sections 3.2.1.3 and 3.2.1.4) was carried out, using the monoclonal antibody 1D9D1, this being an alternative antibody to the affinity-purified polyclonal antibody used to prepare the column. From these results, it appears that HLXOR is represented by a doublet of bands, of approximately 150 kDa and 135 kDa, with the latter being the major band. Both of these bands are positively stained on the corresponding Western blot. It can be seen that there is an additional band with a molecular weight of approximately 50 kDa, highlighted by the arrow. This band is neither a proteolysis nor a degradation product of HLXOR, as a band of this size is not apparent from the Western blot. It is thus likely to represent a contaminating protein.

8.3.1.2 Specific activity

The specific activity was calculated at each stage of the purification, as shown in the table below. Similar starting amounts of liver were used in all purifications.

	Purification 1 (nmol/min/mg)	**Purification 2 (nmol/min/mg)**	**Purification 3 (nmol/min/mg)**
Homogenate	0.0025 52% oxidase	0.0019 50% oxidase	0.0024 55% oxidase
Ammonium sulphate fraction	0.14 15% oxidase	0.12 17% oxidase	0.16 12% oxidase
Immunoaffinity-purified fraction	162 38% oxidase	181 52% oxidase	156 75% oxidase
	Total Yield 0.9 mg	**Total Yield** 0.6 mg	**Total Yield** 0.7 mg

Table 8.3.1

Specific activities were calculated using the urate assay (Section 3.2.4.2). The percentage of enzyme in the oxidase form was also calculated at each stage (Section 3.2.4.2). Total yield of protein (mg) was measured using the Bradford method (Section 3.2.3).

8.3.1.3 UV-visible spectrum

The UV-visible spectrum of immunoaffinity-purified HLXOR is represented below, measured as described in Section 4.2.2.4.

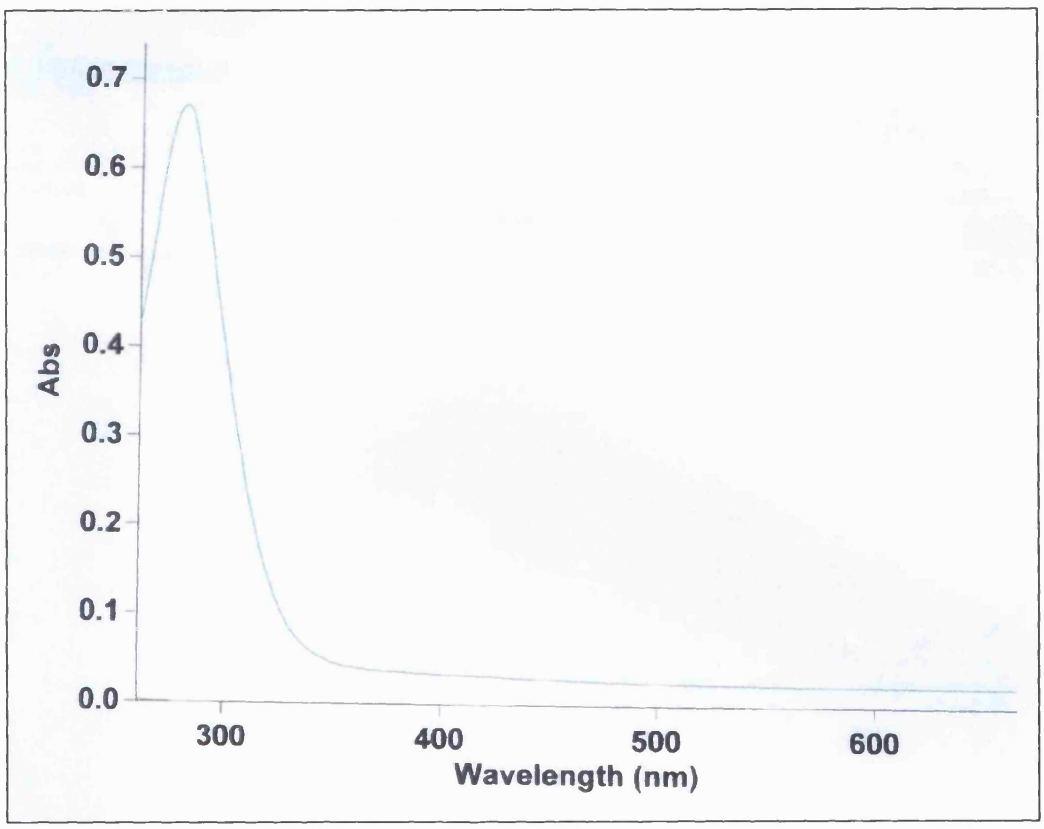


Figure 8.3.3 UV-visible spectrum of immunoaffinity-purified HLXOR

8.3.1.4 N-terminal sequencing

Immunoaffinity-purified protein was subjected to N-terminal sequencing for further identification. The protein was blotted as described in Section 8.2.2.9. The sequencing was kindly carried out by Paul Talbot and Graham Kemp at University of St. Andrews, Scotland (Protein Sequencing Facility).

The major band of 135 kDa (refer to Fig 8.3.2) was sequenced, along with the lower band of approximately 50 kDa, corresponding to a likely contaminant. A BLAST query search was carried out to determine the match of the peptide sequence.

Results:

There were about 200 residues (20 kDa) missing from the start of the sequence at the N-terminal end. The N-terminal sequence of the 135 kDa band through nine cycles was Lys-Lys-Asp-His-Ser (KKDHS).

1 mtadklvffv ngrkvvekna dpettllayl rrklglsgtk lgcgeggcga ctvmlskydr

61 lqnkivhfsa naclapicsl hhvavttveg igstktrlhp vqeriakshg sqcgfctpgi

121 vmsmytllrn qpeptmeeie nafqgnlcrc tgyrpilqgf rtfardggcc ggdgnnpncc

181 mnq**KKDHS** lspslfkpee ftpldptqep ifppellrlk dtprkqlrfe gervtwiqas

241 tlkelldlka qhpdaklvvg nteigiemkf knmlfpmivc pawipelnsv ehgpdgisfg

BLAST query search

KKDHS was matched to:

XDH Human Xanthine Dehydrogenase/Oxidase
Length = 133 kDa
184 KKDHS 188

Human calcium/calmodulin-dependent 3', 5'-cyclic nucleotide phosphodiesterase 1C (HCAM-3)
Length = 71 kDa
616 KKDHS 620
Human hypothetical protein Zap3
Length = 33 kDa
159 KKHDS 163

The lower 50 kDa band contained peptide sequences that were matched to immunoglobulin heavy chains.

8.3.2 Monoclonal immunoaffinity purification

Purification of HLXOR was attempted using immobilised monoclonal antibodies. There are a number of advantages to using monoclonal antibodies over polyclonals. They are essentially available in an unlimited supply, and have the potential to produce a greater overall purification – high-affinity monoclonals are able to bind a large proportion of antigen. In addition, they are desirable because there is less variation between purifications as the monoclonal antibodies are all identical, and bind to the same epitope, compared with polyclonal antibodies, which typically bind to multiple epitopes on the antigen. However, problems with the use of monoclonal antibodies can include low-affinity interactions with antigen, or possible cross-reactions.

For the purpose of immunoaffinity purification, it was necessary to determine which monoclonal antibody clone had a suitable affinity towards HLXOR. A range of anti-(HMXOR) monoclonal antibody solutions were kindly provided (Eurogenetics, Belgium) for testing.

8.3.2.1 Testing specificities of monoclonal antibody clones using Western blotting

Western blotting was performed with three monoclonal antibody clones: 3C9D5, 3E7A6 and 1D9D1. The Western blot results are shown below.

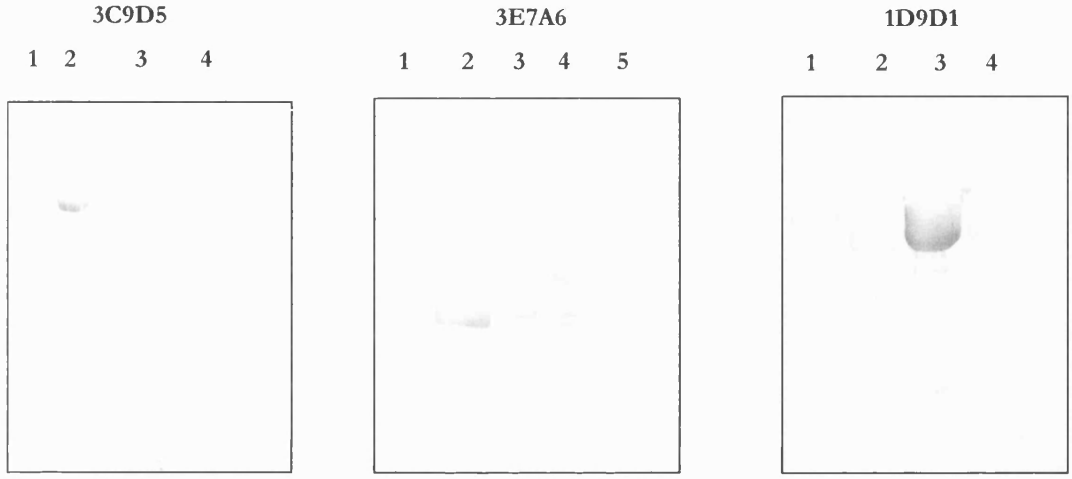


Figure 8.3.4 Western blots using different monoclonal anti-(HMXOR) clones

3C9D5: Lane 1 corresponds to coloured high range molecular weight standards, lane 2 corresponds to purified BMXOR, lane 3 corresponds to human liver ammonium sulphate fraction, and lane 4 corresponds to purified HMXOR.

3E7A6: Lane 1 corresponds to coloured high range molecular weight standards, lane 2 corresponds to purified BMXOR, lane 3 corresponds to purified HMXOR, lane

4 corresponds to commercial BMXOR (Biozyme), and lane 5 corresponds to human liver ammonium sulphate fraction.

1D9D1: Lane 1 corresponds to human liver ammonium sulphate fraction, lane 2 corresponds to purified BMXOR, lane 3 corresponds to purified HMXOR, and lane 4 corresponds to commercial BMXOR (Biozyme).

From these results, it can be seen that the clone 1D9D1 is immunoreactive with human liver XOR and this antibody was therefore chosen to be suitable for immunoaffinity purification.

8.3.2.2 Immunoprecipitation

Two immunoprecipitations were performed (Section 8.2.2.6), using monoclonal antibody 1D9D1 coupled to CNBr-activated Sepharose 4B. The coupled antibody beads were incubated with pure HMXOR and human liver ammonium sulphate fraction, in two separate experiments.

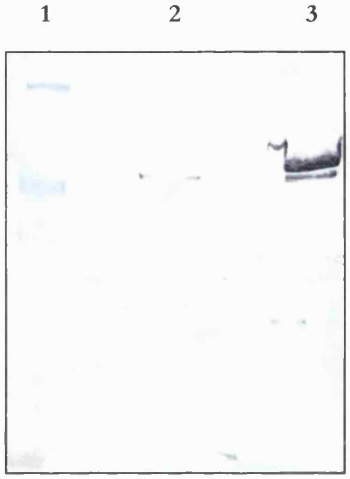


Figure 8.3.5 Immunoprecipitation Western blot

Following elution with 6M urea and centrifugation, samples were concentrated using a Vivaspin concentrator (Vivascience) and electrophoresed on a 7.5% SDS-PAGE gel. Immunoprobing was performed using affinity-purified polyclonal anti-(HMXOR) as the primary antibody.

Lane 1 corresponds to coloured high range molecular weight standards, lane 2 corresponds to precipitated human liver ammonium sulphate fraction, and lane 3 corresponds to precipitated HMXOR.

8.3.2.3 Testing the monoclonal immunoaffinity column with pure HMXOR

A small monoclonal anti-(HMXOR) column (0.5 ml) was prepared (Section 8.2.2.4.1). Purified HMXOR (diluted in PBS) was circulated on the column. 100 mM glycine, pH 2.5, and 25 mM diethylamine, containing 35 mM NaCl, pH 10.5, were tested as eluants. Glycine was investigated as a possibly gentler alternative to diethylamine in view of the lesser number of bonds involved in a monoclonal antigen-antibody complex compared with a polyclonal antigen-antibody complex.

Purified HMXOR (0.5 mg) was diluted in PBS to a final volume of 5 ml. The enzyme solution was circulated onto the column pre-equilibrated in the same buffer for 2-3 h at 4°C. HMXOR elutions and subsequent dialysis were carried out, and incubation with FAD, followed by further dialysis (Section 8.2.2.5) was effected.

8.3.2.3.1 Elution of HMXOR with glycine

Bound protein was eluted from the column with 100 mM glycine, pH 2.5. The trace below shows the elution of bound HMXOR, monitoring the absorbance at 280 nm. The middle peak represents the bound fraction.

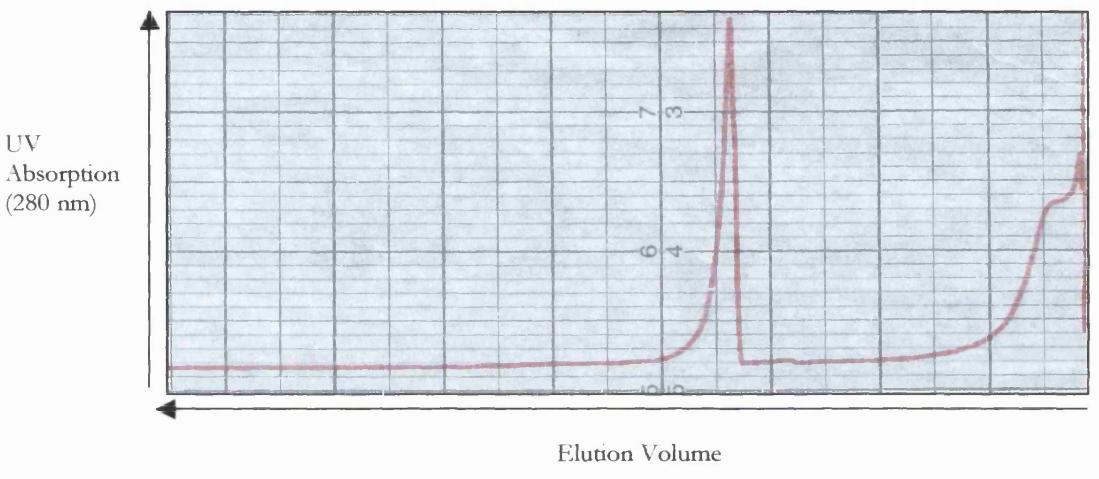


Figure 8.3.6 Elution profile of HMXOR using glycine as eluant

8.3.2.3.2 Elution of HMXOR with diethylamine

Bound protein was eluted from the column with 25 mM diethylamine, containing 35 mM NaCl, pH 10.5. The trace below shows the elution of bound HMXOR, monitoring the absorbance at 280 nm. The peak on the left represents the bound fraction.

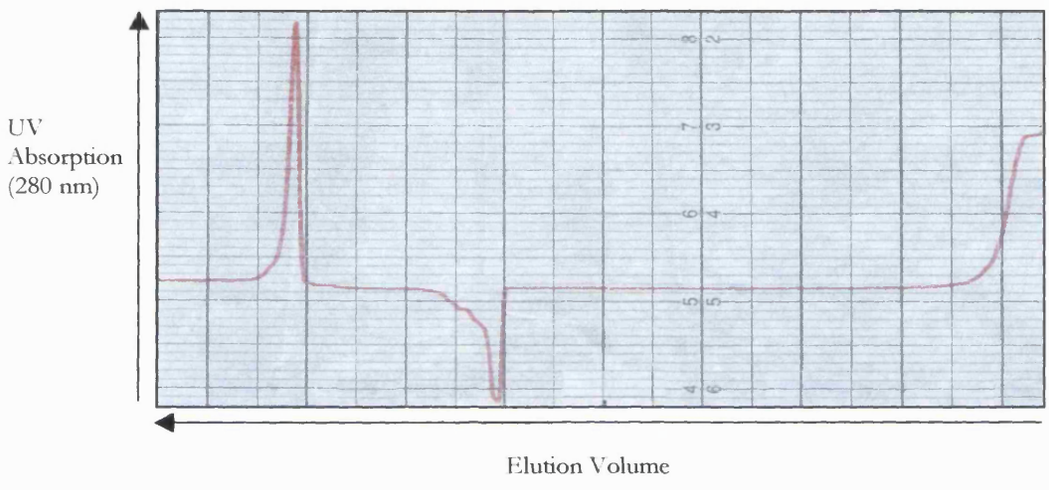


Figure 8.3.7 Elution profile of HMXOR using diethylamine as eluant

8.3.2.3.3 UV-visible absorbance spectra of glycine-eluted HMXOR and diethylamine-eluted HMXOR

The UV-visible spectra of the eluted HMXOR enzymes, measured as described in Section 4.2.2.4, are represented below.

8.3.2.3.3.1 Glycine-eluted HMXOR

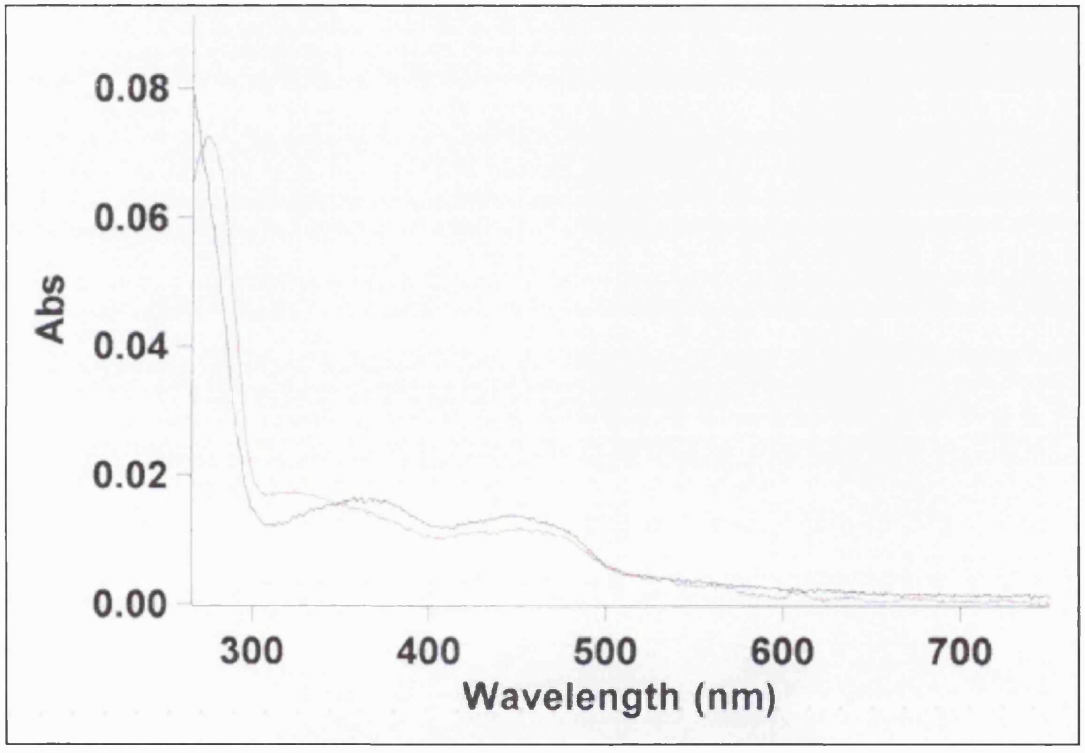


Figure 8.3.8 UV-visible absorbance spectrum of glycine-eluted HMXOR

The red line corresponds to untreated HMXOR, the grey line corresponds to HMXOR, following elution from the column with 100 mM glycine, pH 2.5, and subsequent

incubation with 1 mM FAD (Section 8.2.2.5). The spectra are obtained by dilution of protein with 50 mM Na-Bicine, containing 0.05 M NaCl, pH 8.3.

8.3.2.3.3.2 Diethylamine-eluted HMXOR

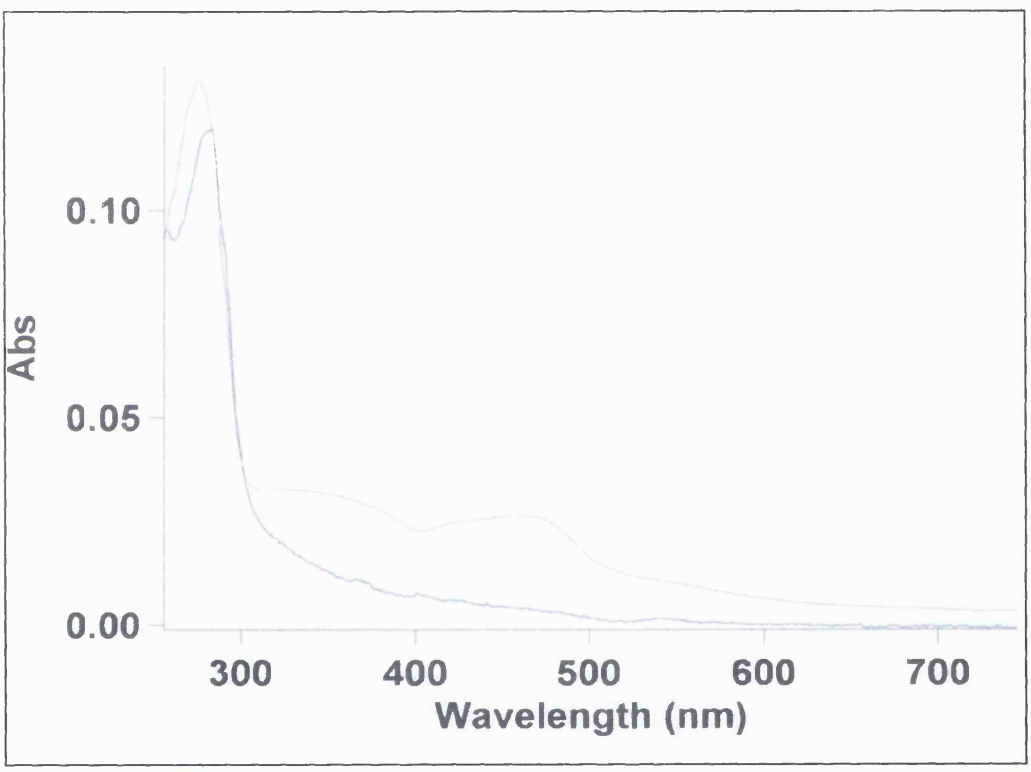


Figure 8.3.9 UV-visible absorbance spectrum of diethylamine-eluted HMXOR

The red line corresponds to untreated HMXOR, the blue line corresponds to HMXOR, following elution from the column with 25 mM diethylamine, containing 35 mM NaCl, pH 10.5, and subsequent incubation with 1 mM FAD (Section 8.2.2.5). The spectra are

obtained by dilution of protein with 50 mM Na-Bicine, containing 0.05 M NaCl, pH 8.3.

8.3.2.3.4 Specific activities of glycine-eluted and diethylamine-eluted HMXOR

The specific activities of HMXOR eluted from the monoclonal anti-(HMXOR) column with the two eluants: 100 mM glycine, pH 2.5 and 25 mM diethylamine, containing 35 mM NaCl, pH 10.5, were determined. These activities were measured following dialysis and incubation with 1 mM FAD (Section 8.2.2.5).

Sample	Specific activity towards xanthine (nmoles/min/mg)	Specific activity towards NADH (nmoles/min/mg)
Untreated HMXOR	72 ± 5	126 ± 14
Glycine-eluted HMXOR	54 ± 3	95 ± 6
Diethylamine-eluted HMXOR	47 ± 5	70 ± 4

Table 8.3.2

Activities towards xanthine were calculated using the urate assay (Section 3.2.4.2). Activities towards NADH were calculated by using the NADH oxidase assay (Section 3.2.4.3). Values are means ± SD (n = 3).

8.3.2.3.5 SDS-PAGE gel of glycine-eluted and diethylamine-eluted HMXOR

Glycine-eluted and diethylamine-eluted enzyme were subjected to SDS-PAGE gel analysis to observe whether molecular weight was altered as a result of immunoaffinity purification, or exposure to either of the eluants.

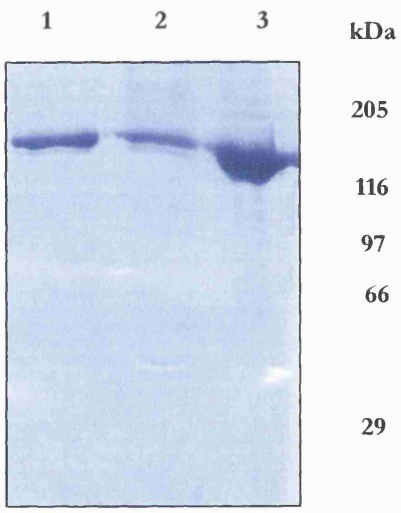


Figure 8.3.10 **7.5% SDS-PAGE gel showing HMXOR following elution from monoclonal anti-(HMXOR) column with glycine and diethylamine**

Lane 1 corresponds to glycine-eluted HMXOR, lane 2 corresponds to diethylamine-eluted HMXOR and lane 3 corresponds to untreated HMXOR (prior to application to the column). The position of high range molecular weight standards are shown alongside the right of the gel.

It can be concluded from the above results that the use of both 100 mM glycine, pH 2.5, and 25 mM diethylamine, containing 35 mM NaCl, pH 10.5, as eluants displaced bound HMXOR from the immunoaffinity column, within a similar time period, producing comparable elution profiles.

The two bound fractions (enzyme eluted with glycine, and enzyme eluted with diethylamine) were subjected to UV-visible scanning to determine whether there had been any changes to the spectral properties as a result of exposure to the eluant. Scanning was performed following incubation with 1 mM FAD, to reconstitute FAD loss as a result of exposure to the eluant. The UV-visible scan of glycine-eluted enzyme (Fig 8.3.7) shows lower absorbance in the region of 310 nm to 400 nm, when compared with the superimposed scan of untreated HMXOR. The remainder of the scan (showing UV absorption above 400 nm) does not seem to have been strongly affected, when compared with untreated HMXOR. The UV-visible scan of diethylamine-eluted enzyme (Fig 8.3.8) appears to be significantly altered. There is considerably lower absorbance in the entire region of 300 nm to 500 nm, when compared with untreated HMXOR. This is despite incubation of enzyme with 1 mM FAD, suggesting either irreversible loss of FAD, or perhaps alteration of the FAD-binding site as well as Fe/S sites of the enzyme, which also contribute to absorbance in this region, resulting in a 'flattening' effect of the scan.

Specific activities towards xanthine and NADH were measured in enzyme eluted from the immunoaffinity column with the two eluants, glycine and diethylamine. Enzyme

was processed for activity assays following dialysis, FAD incubation, and further dialysis to remove excess FAD. The specific activities towards xanthine of the eluted enzyme were lower than that of untreated HMXOR, in both cases, whether eluted with glycine or diethylamine (Table 8.3.2). These activities were 75% and 65% of the original untreated enzyme, when eluted with glycine and diethylamine respectively. This lowered activity could be ascribed to a number of criteria, including protein denaturation or proteolysis, or the formation of inactive enzyme, as a result of loss of Fe/S and/or flavin groups. Incubation with FAD following dialysis was a precautionary measure taken to restore the flavin group, but it may be possible that not all deflavo-enzyme can be reconverted back to its active form by these means. Hille and Massey (1991) reported a restoration of 60 % of original activity upon incubation of deflavo-XOR with free flavin. In terms of NADH-oxidation, which occurs at the FAD site of the enzyme, enzyme eluted with glycine displayed 75% of the original NADH-oxidising activity, and enzyme eluted with diethylamine showed 55% of the original activity, substantiating a greater proportion of deflavo-XOR in the latter preparation. The formation of deflavo-HMXOR is likely to account for the lower specific activities towards xanthine in the immunoaffinity purified preparations. These specific activities suggest that glycine has less of a deleterious effect on the enzyme; a conclusion also supported by the UV-visible scans. Based on these results, it was decided to use 100 mM glycine, pH 2.5, as an eluant in the immunoaffinity purification of HLXOR.

8.3.2.4 Monoclonal immunoaffinity purification of HLXOR

A larger monoclonal immunoaffinity column (5 ml) was constructed, using CNBr-activated Sepharose 4B (Section 8.2.2.4.1). Ammonium sulphate fraction from human liver was first circulated on an uncoupled CNBr-activated Sepharose 4B column, essentially as described in Section 7.2.2.3, to remove non-specific protein. The remaining liver fraction was then subjected to immunoaffinity chromatography (Section 8.2.2.7), using 100 mM glycine, pH 2.5, in elution, in the place of diethylamine. The elution profile of the purification is shown below.

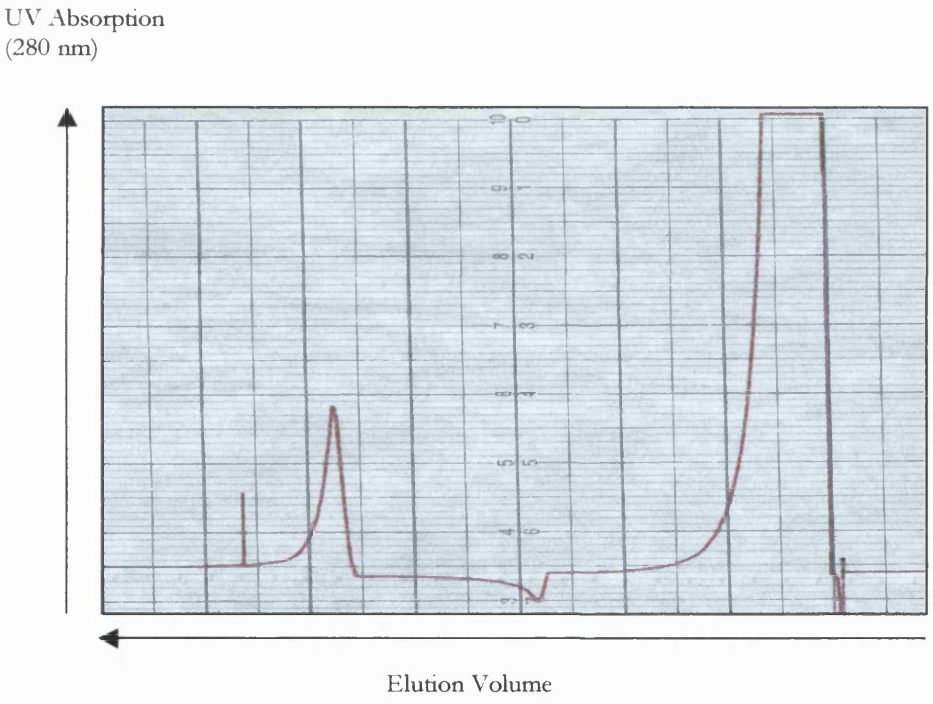


Figure 8.3.11 Elution profile of HLXOR from monoclonal immunoaffinity column

The column eluate was monitored using a UV detector linked to a chart recorder. The sharp peak on the left of the trace represents bound protein eluted with 100 mM

glycine, pH 2.5. The bound fraction was a pale yellow in colour, which seemed to indicate the presence of XOR. The purified milk enzymes, in particular the bovine, are a distinctive yellow-brown, the colour of which can be attributed to the Fe/S and flavin present. Three circulations and subsequent elutions were performed from the same ammonium sulphate liver fraction, and a peak indicating removal of bound protein from the column occurred in each case. Following elution, enzyme was dialysed and incubated with 1 mM FAD (Section 8.2.2.5).

The eluted protein was then circulated on a Protein L-Sepharose column (Section 8.2.2.8). An optimal purification, so described, could be performed over two consecutive days, from tissue homogenisation to completion of column chromatographic steps.

8.3.2.5 SDS-PAGE gel of monoclonal immunoaffinity-purified HLXOR

Following dialysis, the eluted fractions were concentrated using Vivaspin concentrators (Vivascience). The SDS-PAGE gel results are shown below.

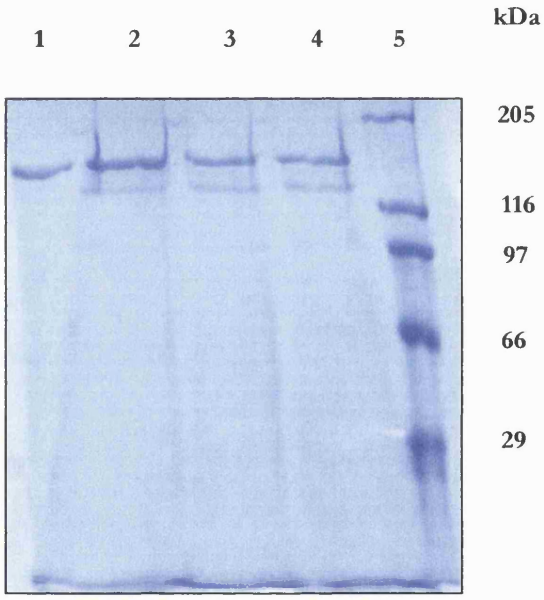


Figure 8.3.12 7.5% SDS-PAGE gel

Lane 1 corresponds to HMXOR, lane 2-4 corresponds to three successive elutions of HLXOR (4 μg) from the monoclonal immunoaffinity column. Lane 5 corresponds to high range molecular weight standards.

The immunoaffinity-purified HLXOR was run alongside HMXOR and BMXOR, and the subsequent SDS-PAGE gel was processed for Western blotting with affinity-purified polyclonal anti-(HMXOR) antibodies.

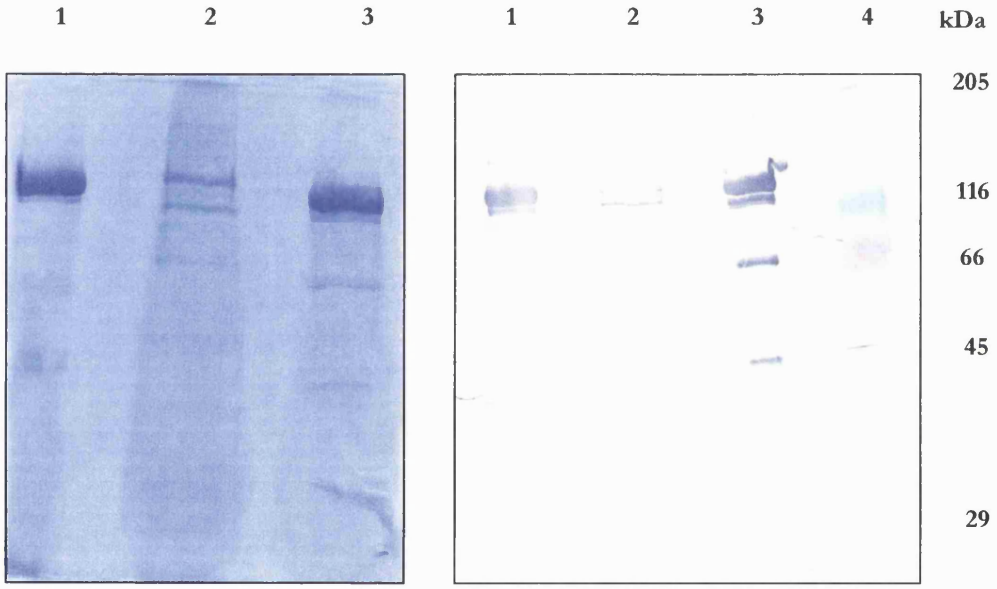


Figure 8.3.13 7.5 % SDS-PAGE gel and Western blot

In both the SDS-PAGE gel and Western blot, Lane 1 corresponds to HMXOR, lane 2 corresponds to HLXOR (4 μg), and lane 3 corresponds to BMXOR. In the corresponding Western blot, lane 4 corresponds to high range molecular weight colour standards.

8.3.2.6 Specific activity

The specific activity of the monoclonal immunoaffinity-purified enzyme preparation was calculated, and is shown in the table below, together with the specific activity of the polyclonal immunoaffinity-purified enzyme (as calculated in Section 8.3.1.2). All purifications used similar starting amounts of liver.

	Purification 1 (nmol/min/mg)	Purification 2 (nmol/min/mg)	Purification 3 (nmol/min/mg)	Total Yield (mg protein)
Monoclonal immunoaffinity-purified HLXOR	145 ± 11 70% oxidase	168 ± 13 55% oxidase	177 ± 9 65% oxidase	1.3 mg
Polyclonal immunoaffinity-purified HLXOR	162 ± 10 38% oxidase	181 ± 12 52% oxidase	156 ± 17 75% oxidase	0.9 mg

Table 8.3.3

Specific activities were calculated using the urate assay (Section 3.2.4.2). Values are means ± SD (n = 3). The percentage of enzyme in the oxidase form was also

calculated at each stage (Section 3.2.4.2). Total yield of protein was estimated using the Bradford method (Section 3.2.3).

8.3.2.7 UV-visible spectra

The UV-visible spectra of immunoaffinity-purified HLXOR and that of purified HMXOR are represented below.

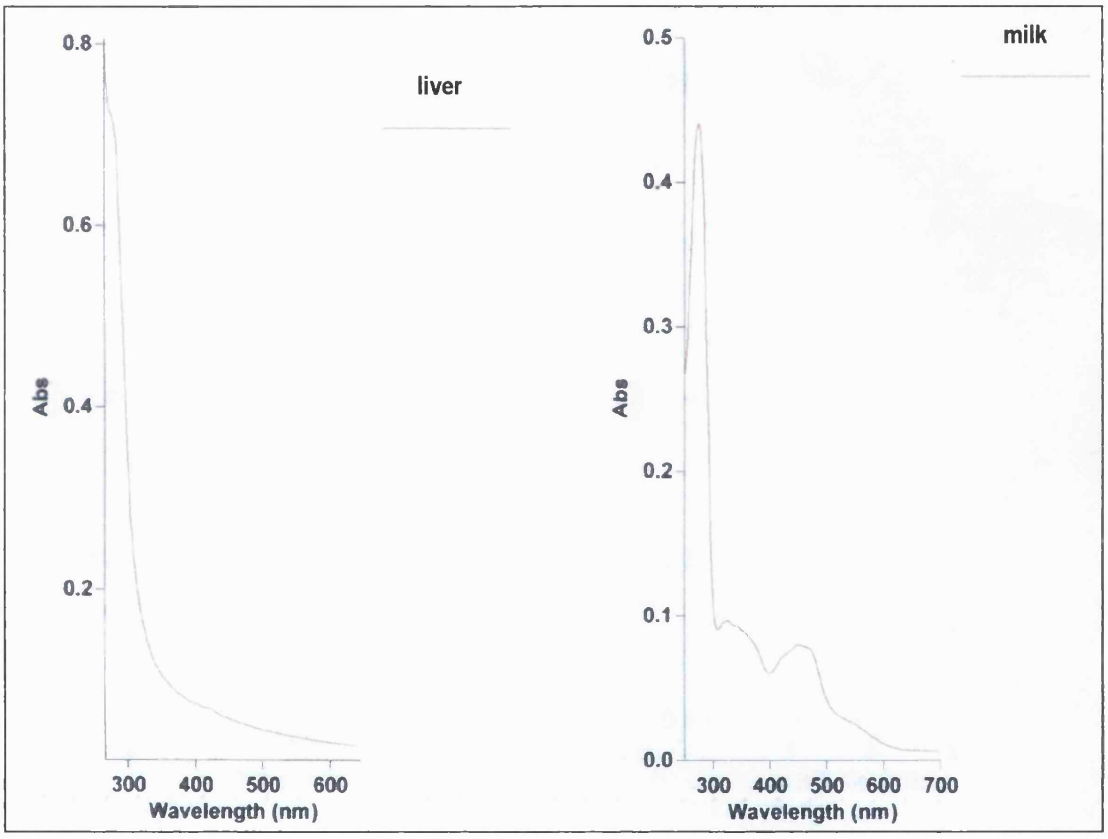


Figure 8.3.14 UV-visible spectra of pure HLXOR and HMXOR

The spectrum of immunoaffinity-purified HLXOR is shown on the left, and the spectrum of pure HMXOR is shown on the right.

8.3.2.8 Coupling monoclonal 1D9D1 anti-(HMXOR) antibody to Protein A-Sepharose

The previously described work involved coupling antibody to CNBr-activated Sepharose 4B to form a monoclonal immunoaffinity column. CNBr-activated Sepharose 4B was selected for use following the success of the immunoprecipitation (shown in Section 8.3.2.2). However, successful earlier purification of HLXOR using a polyclonal immunoaffinity column, described at the beginning of this Chapter, involved the immobilisation of polyclonal antibody to Protein A-Sepharose 4B. It was thus attempted to construct an alternative column by coupling monoclonal antibody 1D9D1 to Protein A-Sepharose, as described in Section 8.2.2.4.2. Coupling was performed with minor modifications to the method used in the coupling of polyclonal antibody to Protein A-Sepharose (Section 7.2.2.1), and a small column (1.5 ml) was constructed. Bound protein was eluted with glycine (as in Section 8.3.2.4). The resulting eluted protein was run on SDS-PAGE gel, which is shown below, together with the corresponding Western blot.

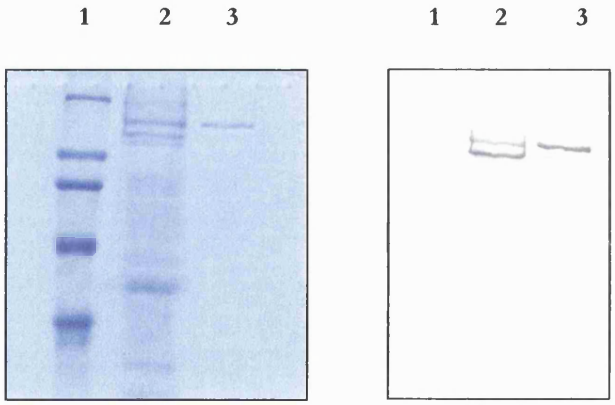


Figure 8.3.15 7.5% SDS-PAGE gel and Western blot

Lane 1 corresponds to high range molecular weight standards, lane 2 corresponds to immunoaffinity-purified protein and lane 3 corresponds to purified HMXOR.

8.4 Discussion

The work in this chapter concerns the immunopurification of human liver XOR utilising both polyclonal and monoclonal anti-(HMXOR) antibodies.

Polyclonal immunoaffinity purification utilised 25 mM diethylamine, containing 35 mM NaCl, pH 10.5, as an eluant. This is the same eluant as that used in the purification of bovine liver XOR. The method gave a reasonably good result, showing two distinct bands on SDS-PAGE analysis, of approximately 150 kDa and 135 kDa, with the major band at 135 kDa (Fig 8.3.1). There was one other band on SDS-PAGE analysis, with molecular weight of approximately 50 kDa. This latter band was not detected by the monoclonal anti-(XOR) antibody (Fig 8.3.2), suggesting that it was not likely to be a proteolysis or degradation product of XOR. Subsequent N-terminal sequence analysis performed on the 135 kDa band substantiated the identity of the enzyme as XOR (Section 8.3.1.4). The sequence of this band was Lys-Lys-Asp-His-Ser, which resulted in a match to human dehydrogenase/oxidase. The sequence of the lower molecular weight band at 50 kDa resulted in matches to immunoglobulin heavy chain. This is in good agreement with the size of the protein, as judged by SDS-PAGE.

The positive sequencing of the 135 kDa XOR band confirms the worth of this method of purification; successful sequencing indicates that there is no blocking of the amino terminus of the enzyme, something that could occur as a result of alternate purification methods. This is particularly common with the use of urea in elution.

The polyclonal immunoaffinity purification method delivered an overall purification of approximately 65000-fold (Section 8.3.1.2). This is significantly higher than the 1600-fold purification achieved by Moriwaki *et al.* (1993), and the 2000-fold purification obtained by Krenitsky *et al.* (1986). However, it must be noted that such an estimate does not take into account the likely presence of intrinsic inhibitors of XOR e.g. purines, in the total liver homogenate, contributing to a lower than true specific activity. In addition, further inaccuracies may be incorporated at this stage with the calculation of total protein in the homogenate. From ammonium sulphate fraction to following immunoaffinity purification there is a purification factor of 975-fold (Table 8.3.1). The proportion of enzyme in the total preparation existing as xanthine oxidase was found to fluctuate at each purification stage. The proportion of oxidase form in the ammonium sulphate fraction is approximately 50 %. In the purified HLXOR preparation, activity due to XO ranged from 38 % to 75 % of the total activity. This differs from the preparation obtained by Krenitsky *et al.* (1986), which was fully converted to oxidase form, reflected in the extensive proteolysis of the enzyme that had taken place. Irreversible conversion of dehydrogenase form to oxidase form is a common occurrence during purification procedures, although the extent of conversion can be minimised by rapid processing, with minimal manipulation of the enzyme (Saksela *et al.*, 1999). Even taking such precautions, tissue preparations have been reported to consistently contain at least 10-15 % oxidase form (McKelvey *et al.*, 1988). Reasons for the dehydrogenase/oxidase ratio variation throughout this purification are unclear. As DTT was kept constant throughout the purification it may be that the proportion of

irreversible xanthine oxidase fluctuates, for example, some irreversible enzyme may be lost between the homogenisation step and the ammonium sulphate precipitation step.

The UV-visible scan of this polyclonal affinity-purified enzyme from liver (Fig 8.3.3) did not resemble that of purified enzyme from milk, for reasons that are discussed at a later stage.

It is not possible to compare fully these results with the polyclonal immunoaffinity purification reported by Saksela *et al.* (1999). These workers did not comment on the degree of purification achieved nor their yield. Their purified preparation exhibited three bands on SDS-PAGE, of 150 kDa, 130 kDa and 85 kDa. The purified HLXOR described in the present work displayed two bands on SDS-PAGE gel of 150 kDa and 135 kDa, with no bands of lower molecular weight due to XOR present. This is likely to be because this preparation is less proteolysed than that obtained by Saksela *et al.*

Affinity methods of purification were employed by Krenitsky *et al.* (1986), who obtained five bands upon SDS-PAGE gel analysis of their purified human liver enzyme, of molecular weights 150 kDa, 135 kDa, 95 kDa, 55 kDa and 38 kDa, with the major band at 95 kDa. Their enzyme preparation was reported to have undergone a moderate degree of proteolysis, although it appeared to retain its physical properties, which were reported to be similar to those of the bovine milk enzymes. This preparation also appears to be significantly more proteolysed than the purified enzyme described here. However, Moriwaki *et al.* (1993) acquired a single band of 150 kDa from their affinity-

purified HLXOR preparation, with no other bands detectable, using both SDS-PAGE and activity staining techniques. Variable degrees of proteolysis reflect differences in the methods of purification and analysis – either before the processing of samples, during purification itself, or on freezing and thawing of the enzyme preparation (Sarnesto *et al.*, 1996). It has been suggested that active enzyme exists in partially proteolysed form in the intact liver tissue (Sarnesto *et al.*, 1996). However, this would be inconsistent with the findings of Moriwaki *et al.* (1993), whereby liver XOR was homogeneous as judged by PAGE. Variable degrees of proteolysis in post-mortem liver maintained under different conditions prior to tissue processing should not be overlooked.

The use of monoclonal antibodies in the isolation of XOR, as opposed to affinity-purified polyclonal antibodies was investigated (Section 8.3.2). Three monoclonal antibody clones (3D9D5, 3E7A6, 1D9D1) were tested for their relative specificities towards HLXOR (in homogenate), and purified XOR enzymes from human and bovine milk (Fig 8.3.4). The testing was performed using Western blot analysis. Of the three clones, 1D9D1 was the most strongly immunoreactive towards liver XOR, with this immunoreactivity being comparable to that towards bovine milk XOR, although highest towards the human milk enzyme. No immunoreactivity towards human liver XOR was detected with either of the other two antibody clones, although both of these were more strongly reactive with enzyme from bovine milk than that from human milk. It is clear, therefore, that all three clones are cross-reactive, albeit to different extents, with XOR from both milk sources. It must be noted that XOR concentration in total liver homogenate is likely to be extremely low, and although maximal amounts of protein

were used in the Western blots, there may not be sufficient enzyme present for reactivity to be seen with a weaker avidity antibody. It is noteworthy that the antibody 1D9D1 displays strongest specificity towards human milk XOR, against which it was raised. This antibody clone was thus chosen to be suitable for the purpose of monoclonal immunoaffinity purification of HLXOR.

Immunoprecipitation was carried out in order to further establish whether the monoclonal antibody immobilised by coupling to CNBr-activated Sepharose 4B was capable of binding human liver XOR present in a crude mixture (Section 8.3.2.2). This was achieved by incubating coupled antibody beads with human liver ammonium sulphate fraction. A faint band of approximately 150 kDa was detected after incubation, equating to purified human liver XOR, and a similarly sized although more pronounced band was detected following incubation with pure HMXOR.

An immunoaffinity matrix of small volume was constructed using 1D9D1 monoclonal antibody coupled to CNBr-activated Sepharose 4B. The resulting column was tested with purified HMXOR to determine the conditions necessary to dissociate the complex (Section 8.3.2.3). In addition, the effect of such a procedure on the enzyme was taken into account, by assessing the activity, electrophoretic and UV-visible properties. HMXOR was circulated and eluted from the column using essentially the same method as that used for polyclonal immunoaffinity purification. However, two elution buffers were tested, these being 100 mM glycine, pH 2.5, and 25 mM diethylamine, pH 10.5 (containing 35 mM NaCl). Both buffers promoted dissociation of antigen-antibody

complex, producing similar elution profiles. Neither buffer produced any changes in the electrophoretic properties of the enzyme (Fig 8.3.10). The most notable differences were observed in the UV-visible absorbance spectra. Diethylamine appeared to adversely affect the absorbance spectrum of HMXOR, producing a 'flattened' scan, apparently lacking absorbance attributable to the Fe/S and flavin groups of the enzyme (Fig 8.3.9). Glycine resulted in a scan more closely resembling that of untreated HMXOR (Fig 8.3.8). Elution with diethylamine more adversely affected the specific activity of the enzyme than did elution with glycine. For these reasons, glycine was selected for use as eluant in the purification of HLXOR.

Subjection of human liver ammonium sulphate fraction to monoclonal immunoaffinity chromatography produced a sharp increase in protein upon elution with glycine, indicating removal of bound protein from the column matrix. The pooled bound and eluted protein fractions were then applied to a Protein L-Sepharose column to remove residual immunoglobulin, the presence of which was observed with polyclonal immunoaffinity chromatography, as evidenced by SDS-PAGE patterns and N-terminal protein sequencing. Such immunoglobulin may derive from an endogenous source, present in the human liver homogenate from the beginning of the purification, possibly co-existing with XOR as an immune complex. Anti-(XOR) antibodies have been reported to occur in human serum (Oster *et al.*, 1974; Bruder *et al.*, 1984; Harrison *et al.*, 1990; Ng *et al.*, 1990; Benboubetra *et al.*, 1997). It is proposed that the IgM anti-(XOR) antibodies may play a protective role in the removal of circulating and potentially

damaging XOR (Benboubetra *et al.*, 1997). It is unclear whether such antibodies and corresponding immune complexes exist in tissues, however.

A second source of immunoglobulin contaminating the purified enzyme preparation may be degradation or leaching from the immunoaffinity column itself. Degradation seems unlikely, as it is known that antibodies as a group are highly stable, particularly resistant to proteolytic attack, and are cleaved selectively by only a limited number of enzymes. However, leaching of the antibody from the column is a common problem with CNBr-activated Sepharose, as the covalent bonds formed between the ligand and the matrix are relatively labile. Although such leakage of antibody was not detected during SDS-PAGE analysis, when testing the monoclonal column with purified HMXOR, it was decided to incorporate a further 'subtractive' immunopurification step using Protein L-Sepharose, as a precaution. The presence of anti-(XOR) antibody in the immunopurified XOR preparation is particularly undesirable as it may inhibit by binding to the enzyme, producing an inaccurate calculation of specific activity. Such an effect has been observed with affinity-purified anti-(human XOR) antibodies displaying partial inhibition of XOR enzymic activity (Benboubetra *et al.*, 1997).

SDS-PAGE analysis of the final purified protein resulted in two distinct bands, with the major band at approximately 155 kDa and the minor band at 135 kDa (Fig 8.3.12), showing a similar pattern to the doublet of 150 kDa and 135 kDa obtained with polyclonal immunoaffinity purification. The observation that the major band is equivalent to the higher molecular weight band of the doublet differs from polyclonal-

affinity purified HLXOR where the major band is of 135 kDa as opposed to 150 kDa. This could be because the monoclonal affinity-purified HLXOR is less proteolysed than the polyclonal affinity-purified preparation. Hellsten-Westing (1993) identified three main bands on SDS-PAGE attributable to human liver XOR, with molecular weights of 155 kDa, 143 kDa, and 95 kDa, following affinity purification using monoclonal antibody. The first two bands obtained by Hellsten-Westing are similar to those obtained in the results here, although no lower band at 95 kDa was apparent. As discussed previously, different proteolytic states of the original liver samples are clearly a potentially complicating factor.

Western blotting of the monoclonal immunopurified protein using polyclonal affinity-purified anti-(HMXOR) antibody produced staining of both the 155 kDa and 135 kDa bands. The immunopurified HLXOR was electrophoresed alongside BMXOR and HMXOR, and it is interesting to note that there are apparent differences in molecular weight between the three XOR enzymes. HLXOR is represented by a distinct doublet of bands on SDS-PAGE gel, at 155 kDa and 135 kDa. HMXOR typically displays a single band on SDS-PAGE gel, corresponding to a molecular weight of approximately 150 kDa, although when slightly proteolysed, a second band is commonly observed corresponding to approximately 135 kDa, similar to that observed with HLXOR in this study. This has been observed within the laboratory and has been reported by Abadeh *et al.* (1992). It can be seen from the SDS-PAGE results in Figs 8.3.12 and 8.3.13 that the two human enzymes run essentially as the same molecular weight.

Bovine milk XOR, on the other hand, appears to differ slightly in molecular weight, as judged by SDS-PAGE. The major band of the bovine enzyme corresponds to a fractionally lower molecular weight than that of HMXOR, of approximately 147 kDa (Godber, B., Ph.D. Thesis, 1998). However, it appears that the three enzymes do share similar patterns of degradation. A commercially obtainable form of BMXOR produces multiple degradation bands on a gel, routinely observed within our laboratory. The molecular weights of these bands bear similarities to a proteolysed preparation of human milk enzyme. Sarnesto *et al.* (1996) comment that the cleavage of purified HMXOR and enzyme in tissue homogenates is not random, due to the observation that similarly sized fragments are consistently apparent on SDS-PAGE analysis and Western blotting. As previously mentioned, it cannot be ruled out that the variations in number of, and apparent sizes of minor bands seen on SDS-PAGE, might reflect variable extents of proteolysis of the enzyme.

Nevertheless, differences between the mobility of bovine and human milk enzymes have long been observed in our laboratory, and these cannot themselves be explained in terms of tertiary structure of the proteins (Godber B., Ph.D. Thesis, 1998). The amino acid sequences of BMXOR (Berglund *et al.*, 1996) and HLXOR (Ichida *et al.*, 1993) have been reported and are very similar, although the liver sequence contains 143 amino acid changes. The sequence of human mammary gland epithelial cell XDH has been determined (Pearson, A., Ph.D. Thesis, 2001) and is identical to those sequences already determined for human liver and small intestine (Ichida *et al.*, 1993; Saksela & Raivio, 1996; Yamamoto *et al.*, 2001). The primary sequence of HMXOR is also identical to

that determined for BMXOR, and it appears that differences between the two enzymes are mainly in the 3-dimensional structure of HMXOR, which is lacking molybdenum cofactor (Pearson, A., Ph.D. Thesis, 2001). If the differences in molecular weight were observed under non-denaturing, native conditions, they might be explained in terms of enzymic structure, for example, HMXOR will have a more open conformation due to the lack of molybdenum cofactor (Godber *et al.*, 1997; Pearson, A., Ph.D. Thesis, 2001). However, as separation by SDS-PAGE is dependent on molecular size alone, and the net charges of all proteins are made constant, it is difficult to explain these observations. In view of this, the difference in the apparent molecular weights of the human and bovine enzymes pose a quandary that is very difficult to explain.

There is not much difference observed between the specific activities towards xanthine of the polyclonal and the monoclonal immunopurified enzymes, nor in the yields obtained using the two methods (Table 8.3.3). The mean specific activity of the purified liver enzyme using monoclonal immunoaffinity purification (166 nmol/min/mg) is significantly higher than the specific activity of the enzyme purified by Moriwaki *et al.* (1993) (0.96 nmol/min/mg), using a range of non-immune affinity methods. Moriwaki *et al.* used essentially the same procedure to calculate enzyme activity towards xanthine and it is unclear why the specific activity should differ so greatly. Such disparity may be attributed towards differences in the purification procedures used, and if this is the case, one may conclude that immunoaffinity methods are less detrimental towards the activity of the enzyme than non-immune methods of purification. On the other hand, the difference in activity may be due to isolation of active enzyme using affinity methods of

Moriwaki *et al.*, whereas the immunoaffinity methods used in this study do not distinguish between active and inactive forms of enzyme. This, and the specific activity of HLXOR in relation to the respective specific activities of the two milk XORs will be discussed further in the next Chapter.

The UV-visible spectrum of the monoclonal immunopurified enzyme was compared with that of purified HMXOR (Fig 8.3.14). However, the spectrum of the liver enzyme was disappointing in that it did not show the distinctive peaks at 350 and 420 nm, suggesting that the flavin and Fe/S sites had been affected as a result of the purification. Although some yellow-brown colour (usually indicative of both flavin and Fe/S groups) was observed in the column eluate, this did not appear to be reflected in the scan. HLXOR was incubated with FAD following dialysis, to reconstitute the enzyme if flavin concentration was low, as a result of exposure to the eluant glycine. In addition, during earlier experiments subjecting purified HMXOR to immunoaffinity chromatography and elution with glycine there were no such detrimental effects to the enzyme apparent from the UV-visible spectrum. However, there may be some alternate damage to the structure of the immunopurified liver enzyme, accounting for the irregularities in the UV-visible properties.

As a final experiment, it was attempted to conjugate monoclonal antibody to Protein A-Sepharose (Section 8.3.2.8), as was performed with polyclonal anti-(HMXOR) antibody, described at the beginning of this Chapter. This was performed in order to compare the relative efficacy of the resulting purification with the purification achieved using

monoclonal antibody coupled to CNBR-activated Sepharose 4B, which has already been established to be a viable method. It is evident from the numerous bands observed upon SDS-PAGE of the immunopurified protein that the purification was not effective. Although XOR was evident in the bound fraction, as determined by the Western blot, several other proteins co-purified. This could indicate some problem with the antibody coupling, or perhaps the orientation of the antibody did not promote a stable interaction with the ligand. It is known that mouse monoclonals have a far lower affinity towards Protein A than the rabbit polyclonals used previously. Thus, the low affinity of the antibody may result in a weak coupling with the Protein A matrix, leading to leaching of antibody during column usage, and an inefficient purification.

Considering the two immunoaffinity methods described in this Chapter, employing both polyclonal and monoclonal anti-(HMXOR) antibodies, it is reasonable to conclude that both are effective and rapid means of XOR purification, as opposed to non-immune affinity procedures. Enzyme purified using monoclonal antibodies appeared to be purer than that purified with polyclonal antibodies. Thus, for all further studies requiring purified HLXOR, monoclonal immunoaffinity-purified enzyme was chosen.

9. Human liver xanthine oxidoreductase (HLXOR): specific activity, immunolocalisation and crystallisation

9.1 Introduction

9.1.1 XO and liver disease

It is known that hereditary xanthinuria is characterised by undetectable levels of xanthine oxidoreductase (XOR) activity in patients. There is, however, little known about the extent and nature of variations of XOR activity in human individuals, nor about the significance of such variation in drug metabolism (Guerciolini *et al.*, 1991). One investigation attempted to study functional variation of xanthine oxidase (XO) activity by measuring the urinary metabolites of caffeine, which is partly metabolised by XO (Grant *et al.*, 1983). This study found an approximately 2.8-fold variation among a total of 68 subjects studied, with no indication of the existence of subgroups within this population sample. A more recent study (Guercolini *et al.*, 1991) used a sensitive radiochemical assay to study activity in 189 hepatic tissue samples, taken from patients undergoing partial hepatectomy or open liver biopsy. Interestingly, the results indicated an average 21 % higher XO activity in samples taken from male patients than in those from female patients. In patients with normal liver function, results suggested the presence of a subgroup of samples with relatively low XO activity in 21 % of male patients and 27 % of female patients. The authors proposed that these observations

may be associated with individual variation in the biotransformation of drugs metabolised by XO.

As a potent generator of ROS and free radicals, XOR has been implicated in the pathogenesis of many diseases, particularly ischaemia-reperfusion injury (McCord, 1995). XO levels in human serum are almost undetectable under normal conditions. However, increases have been reported in numerous pathological states, most notably those affecting the liver, in which the hepatocytes, sinusoidal endothelial cells and Kupffer cells are reportedly rich in the enzyme (Wiezorek *et al.*, 1994). Elevated levels of human serum XO have been found in several cases of liver damage, such as viral hepatitis (Shamma'a *et al.*, 1965; Ramboer *et al.*, 1972; Wolko & Krawczynski, 1974; Giler *et al.*, 1975; Yamamoto *et al.*, 1996), in alcoholism (Grattagliano *et al.*, 1996), and following halothane anaesthesia (Giler *et al.*, 1977). Increased XO activity in serum has been observed after tourniquet ischaemia in patients undergoing reconstructive surgery, whereby effluent blood was evaluated following reperfusion (Friedl *et al.*, 1990). Reperfusion during liver transplantation has also been found to be associated with release of XO, as well as hypoxanthine and xanthine, from the liver into the circulation (Pesonen *et al.*, 1998). Circulating XO has been suggested to cause oxidative damage at other sites through the production of ROS (Yokoyama *et al.*, 1990). Higher levels of XO were measured in the plasma of preterm neonates (Supnet *et al.*, 1994). This is of particular significance as liver damage is very common among extremely premature infants. Elevated levels of XO are also found in the plasma of hypoxic patients with adult respiratory distress syndrome (Grum *et al.*, 1987). A recent study has established

levels of XO in the sera of patients with various inflammatory and autoimmune rheumatic diseases to be up to 50-fold higher when compared to the sera of healthy donors and patients with non-rheumatic diseases (Miesel & Zuber, 1993). The determination of XO levels in sera could have a clinical value, at least in the evaluation of liver diseases. Various tests have been designed for this purpose, including those based on activity assays (Yamamoto *et al.*, 1996) and ELISA (Batelli *et al.*, 1999). It has been suggested that release of XO into the serum could occur as a result of hepatic cell damage, or severe liver necrosis (Batelli *et al.*, 1996). The administration of ricin induced liver necrosis in rats, causing liver XO to be markedly increased, although serum XO remained the same. On the other hand, treatment with saporin resulted in elevated serum XO, but not in levels of liver enzyme, suggesting functional differences in necrotic liver lesions (Batelli *et al.*, 1996).

Batelli *et al.* (2001) investigated, by ELISA, the level of XO in the serum of patients with various liver disorders, including cirrhosis, chronic viral hepatitis and cholestasis (a disorder of the biliary tract). They found XO levels to be significantly higher in all patients, compared with controls. Cholestatic patients had serum XO values higher than those of patients with cirrhosis or chronic hepatitis. There appeared to be no correlation between serum XO levels and evidence of hepatocyte necrosis in these patients, however, leading to the proposal that the enzyme is either not released during hepatocyte injury, or it is released, but only in the very early stages of damage.

Primary biliary cirrhosis (PBC) is a chronic autoimmune-mediated biliary disease, resulting in a loss of bile ducts. It is characterised by the spontaneous destruction and obliteration of septal and intrahepatic bile ductules, associated with an infiltration of plasma cells and lymphocytes in the portal tracts, hepatitis, fibrosis, and slowly progressive intrahepatic cholestasis, ending with established biliary cirrhosis (Kaplan, 1987; Moreno-Otero *et al.*, 1989). The disease is believed to be the result of an altered autoimmune response mediated by T cells, or by disease-specific antimitochondrial autoantibodies, thought to be less likely (James *et al.*, 1983). A connection between the accumulation of free radicals, and the process of lipid peroxidation, has been implicated in PBC (Ono *et al.*, 1991), in addition to other hepatic disorders, including alcoholic hepatitis and hepatocarcinogenesis (Marklund *et al.*, 1982; Klein *et al.*, 1983; Kubota *et al.*, 1985).

Chronic cholestasis, a feature of PBC, results in the accumulation of cytotoxic bile acids in the liver, thought to be the major cause of liver necrosis and subsequent liver fibrosis (Poli *et al.*, 1993; Parola *et al.*, 1996). Schimpl *et al.* (2000) investigated the hepatic XO and XDH activities in the livers of chronic cholestatic rats. Their results showed a significant increase in XO activity, leading to the hypothesis that the oxidative hepatic parenchymal cell damage seen in this disease might at least be partially initiated by XO-generated ROS. Treatment with allopurinol attenuated hepatic XO activity, exerting a hepatocellular protective effect.

9.1.2 Localisation of liver XOR

Kooij *et al.* (1992) investigated the localisation of both the dehydrogenase and oxidase forms of XOR in post-mortem human liver, using an activity-based detection method. Activity was higher in the periportal sinusoidal cells of the liver lobule, whereas the pericentral area was less intensely stained. In addition, activity was observed in the hepatocytes of both the pericentral and periportal areas. Individual variation in the XOR activity of the human livers measured in this study was also apparent, confirming the earlier findings of Guerciolini *et al.* (1991).

Immunohistochemical localisation of XOR has been reported in various tissues from various animal species, reviewed by Kooij (1994) and Moriwaki *et al.* (1997) but there have been fewer reports on the localisation in human tissues. These reports, particularly those concerning the liver, show conflicting results. The immunohistochemical localisation of human liver XOR was examined in 1993 by Moriwaki and co-workers. They used polyclonal antibodies raised against HLXOR and an avidin-biotin-peroxidase detection system. XOR was detected in the cytoplasm of hepatocytes and endothelial lining cells, but not in the bile ducts. They confirmed the cytoplasmic location of the enzyme, first reported from the biochemical data of Jarasch *et al.* (1981). A second study by the same group, based on essentially the same method, found XOR localisation in the cytoplasm of hepatocytes once again, with more intense staining in the pericentral and periportal regions than the mid-zonal area, and in the sinusoidal cells. Staining of the bile duct was also seen, in contrast to the previous findings (Moriwaki *et al.*, 1996b).

In a more recent study, Linder *et al.* (1999), using an immunohistochemical method with a polyclonal antibody raised against HMXOR, detected a strong lobular gradient of XOR protein. Staining was evident in the cytoplasm of periportal hepatocytes, in the Kupffer cells of some liver samples, and absent in the perivenous region. Bile duct epithelia were negative, as well as portal vein and hepatic artery endothelium. These findings are partly at variance with the previous findings of Moriwaki *et al.* (1996b). The staining of XOR in Kupffer cells, which Linder *et al.* found in one-third of liver samples was not reported by Moriwaki *et al.* (1996b). Linder *et al.* went on to propose that XOR expression in the Kupffer cells, which are the resident macrophages of the liver, could either be the result of induction in these cells, or the uptake of protein from the circulation. The failure of Linder *et al.* to find staining in the bile ducts is also at variance with Moriwaki *et al.* (1996b).

The disparity between these immunohistochemical results in human liver could well be the result of the varying natures of antibodies used in detection. There is a considerable need for reliable and consistent information regarding XOR distribution in the liver, using a well-characterised and highly specific antibody. Such information will provide us with greater understanding of the pathophysiological role of this enzyme.

9.1.3 Crystallisation of XOR

Small crystals of bovine milk XO were obtained as early as 1954, during the purification of the enzyme (Avis *et al.*, 1954). They were not, however, of a sufficient quality to allow analysis. Diffraction to 2.1 Å was briefly reported from crystals of bovine milk XO in 1993 (Eger *et al.*, 2000) but it was only recently that the solution of the crystal structures of both BMXO and BMXDH (complexed to the inhibitor salicylate) to 2.1 Å and 2.5 Å respectively, were presented (Enroth *et al.*, 2000). In this report, Enroth and colleagues described the major three-dimensional changes occurring in the proteolytic conversion of the dehydrogenase to the oxidase form of the enzyme. Rat liver XO crystals have been produced, diffracting to 2.6 Å, although a solution has not yet been reported for these (Carvalho *et al.*, 1998).

Crystals of human milk XOR, in its demolybdo form, diffracting to 3.5 Å, have been very recently obtained (Pearson, A., Ph.D. Thesis, 2001). This is the first report of the crystal structure of both human XOR, and of a demolybdo form of XOR.

Prior to the elucidation of the crystal structure of XOR, that of aldehyde oxidoreductase (MOP) from *D. gigas*, which shows a 50 % sequence identity with XOR, was the only such information from a representative of the XOR family (Thoenes *et al.*, 1994; Romao *et al.*, 1995). MOP contains a molybdopterin and two Fe/S cofactors, although it lacks the FAD domain. Study of its structure allowed understanding of the role of the metal coordination site in catalysis and the proposal of a catalytic mechanism for XOR. The crystal structures of other XOR family members followed. These included aldehyde

oxidoreductase (MOD) from *D. desulfuricans* (Rebelo *et al.*, 2000), carbon monoxide dehydrogenase (CODH) from *O. carboxidovorans* (Dobbek *et al.*, 1999) and the demolybdo CODH from *H. pseudoflava* (Hanzelmann *et al.*, 2000). Together these structures have succeeded in providing us with details of the environment and arrangement of the various prosthetic groups, as well as contributing to knowledge of structure-based mechanisms of XOR activity.

The work in this Chapter investigates the specific activity of human liver XOR, isolated using the monoclonal immunoaffinity purification described in the previous Chapter, and relates this activity to that of enzymes from bovine and human milk. Quantification of XOR protein in human liver homogenate, using ELISA, is also attempted. XOR activity in homogenates of human primary biliary cirrhotic liver is then discussed, and contrasted with that in normal human liver. Immunohistochemical staining is employed using 1D9D1 monoclonal antibody to detect localisation of XOR in human liver sections. Finally, the crystallisation of HLXOR is reported.

9.2 Materials and Methods

9.2.1 Materials

Frozen human liver (normal and primary biliary cirrhotic) was obtained from the Royal Free Hospital, London, as before. Blocking reagent used in tissue immunostaining was obtained from Roche Diagnostics, Germany. Mounting medium (Vectashield) was obtained from Vector Laboratories Inc., Burlingame, California. Terasaki plates used in crystal screening were obtained from Hampton Research. All other chemicals were obtained from Sigma-Aldrich, Poole, Dorset, unless stated otherwise.

9.2.2 Methods

9.2.2.1 Native (non-reducing) PAGE

6 % and 7 % gels and buffers were prepared as described as in Section 3.2.1.1, but with the omission of SDS and 2-mercaptoethanol. The concentration of acrylamide: bisacrylamide was varied accordingly. Samples were diluted in 25 mM Na-phosphate, pH 7.4, and mixed 1:1 with sample buffer (Section 3.2.1.1) and loaded onto the gels. Electrophoresis was carried out at 75 V, until samples had passed through the stacking gel, and then at 200 V until the dye had reached the bottom of the gel, upon which electrophoresis was complete. Gels were stained for protein, again as described in Section 3.2.1.1.

9.2.2.1.1 XOR activity staining

The gel was incubated for 10-30 min, in 50 mM Tris-HCl, pH 7.5, containing 20 mM hypoxanthine and 3 mM nitroblue tetrazolium (NBT). To remove substrate, and prevent further development of staining, the gel was rinsed several times in distilled water.

9.2.2.2 ELISA for the determination of XOR protein

This was carried out as described in Section 3.2.5.1.

9.2.2.3 Immunostaining of liver sections

Paraffin-embedded sections were prepared from frozen liver and mounted on glass slides. This was kindly performed by colleagues at the University of Bath.

Sections were dewaxed by placing slides in xylene with two changes, for 3 min each. Rehydration was effected by immersion in a succession of graded alcohols (100 % ethanol, two changes, 95 % industrial methylated spirit, 90 % industrial methylated spirit, 70 % industrial methylated spirit, 1 min each), followed by distilled H_2O. The slides were incubated in pre-warmed Na-citrate/citric acid, pH 6.5, at 37 °C, for 30 min, for antigen retrieval. They were then rinsed in PBS (three times, 15 min/wash, shaking gently) and 0.5 % Triton X-100 (diluted in PBS) was added for permeabilisation. Slides were rinsed as before. Blocking was performed with 10 % heat-inactivated horse serum/6 % BSA, or Blocking reagent (Roche Diagnostics), for 1 h 45 min, while shaking gently.

Primary antibody (1D9D1, diluted 1:100 in blocking solution) was added to cover the surface of slides, which were incubated overnight, at 4 °C. Washes were carried out as before. Secondary antibody (Anti-Mouse IgG (whole molecule) FITC conjugate (developed in rabbit); 1:100 dilution in blocking buffer as before) was added to cover slides. Following the addition of this light-sensitive antibody conjugate the slides were kept in the dark. Slides were incubated with antibody for 2 h at room temperature. Washes were performed as before. A coverslip was mounted on top of the sections on each slide, using mounting medium (Vectashield), and sealed round the edges using clear nail varnish.

Slides were viewed with the use of a confocal laser scanning microscope. Settings: Argon laser 488 nm wavelength, times 63 oil immersion lens with s 63 (NA 1.40). A FITC (Cy 2) filter was used.

9.2.2.4 Crystallisation of XOR

A protein crystal is a fairly open three-dimensional lattice, in which each repeating motif is a single protein molecule or group of molecules. Water represents a major proportion of the crystal volume, and the water molecules surrounding the protein are well organised, forming hydrogen bonds with the protein and each other. Only a small portion of the protein surface is in contact with other protein molecules. Interactions involved in contacts between protein molecules are similar to those believed to stabilise the protein structure, such as hydrogen bonding, hydrophobic and ionic interactions, and less commonly, metal coordination. As a result, the crystals are soft and very sensitive to environmental factors such as humidity.

Crystallisation of proteins and other biological macromolecules occurs from an aqueous solution containing a variety of cosolutes, the major cosolute being the precipitant. The crystallisation process involves manipulation of the protein's solubility, and the maintenance of a single and stable state of homogeneous content and conformation. There are three phases of crystallisation: nucleation, growth and cessation of growth. The process is dependent on the formation of a supersaturated solution from which the desired molecule will precipitate in a crystalline or amorphous state.

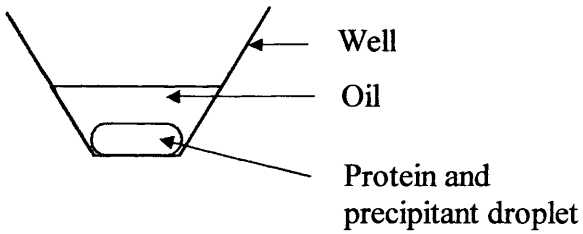
There are many variables influencing crystallisation, such as purity and concentration of protein, temperature, pH and buffer, precipitant type and concentration, biological or enzymic contamination and the presence of chelators, to name but a few. The protein to be crystallised must be of the highest purity obtainable, soluble, stable and clean.

Preliminary crystallisation experiments used the Hampton Research Crystal Screen (Crystal Screen 1), which is based on the sparse matrix screen described by Jancarik & Kim (1991). This is the most widely used general screen and involves 50 different conditions, using a wide range of precipitants, buffers and salts which, combined, provide the most common crystallisation conditions recorded at that time. This procedure makes it more likely to obtain a positive result, or 'hit' with most proteins. A 'hit' can be anything from a crystalline precipitate to the formation of stars, or spherulites, to the appearance of a single crystal. Once a 'hit' is found, further screening experiments can be designed to optimise the conditions to promote crystallisation. In addition, the absence of any 'hit' under a particular condition can provide us with more information about the solubility of the protein of interest.

The method used here is known as the "microbatch" method, where a droplet of protein solution is immersed in paraffin oil, which slows the evaporation of the solution. Very small volumes can be used (1 μl), making the method ideal in the initial stages of screening. This approach involves the addition of precipitant or changing pH until the protein comes to the limit of its solubility, and enters a state of supersaturation. If successful, a few nuclei are generated from which crystals will grow. All conditions in such a method are static, with protein concentration decreasing over time. Growth stops when the solution becomes so depleted of protein that no further growth can occur, or the surface of the growing crystal becomes contaminated with impurities, ending growth.

Purified HLXOR (1.5 mg/ml) used in trials was in 50 mM Na-Bicine, pH 8.3 (containing 0.05 M NaCl, 1 mM EDTA and 4 mM DTT). A Terasaki microbatch plate (Hampton Research) was used, containing 72 cone shaped wells, each holding up to 20 µl. The wells were filled with paraffin oil (liquid colourless light paraffin; 15 µl). Purified HLXOR (1 µl) solution (pre-incubated with 10 mM DTT for 1 h) was added to each well, with care taken to pipette under the oil surface. Each precipitant solution (1 µl) was added to each well, and the drop was allowed to sink to the bottom. Mixing of protein and precipitant solution was performed with pipetting. The plate was covered and stored in a quiet room, at 20 °C. The plate was inspected at daily intervals for recording observations.

The diagram below illustrates a microbatch under oil experiment.



9.3 Results

9.3.1 Specific activity of immunoaffinity-purified HLXOR and BLXOR, affinity-purified HMXOR and BMXOR

The specific activities of HLXOR purified by the monoclonal immunoaffinity chromatography described in the previous Chapter (Section 8.3.2.4), and BLXOR purified by polyclonal immunoaffinity chromatography (Section 7.3) were compared with the activities of HMXOR and BMXOR, purified within the laboratory using affinity methods (detailed in Chapter 3).

Enzyme	Specific activity towards xanthine (μmol/min/mg)
HLXOR	0.17 ± 0.02
BLXOR	0.72 ± 0.04
HMXOR	0.068 ± 0.005
BMXOR	1.38 ± 0.9

Table 9.3.1 Specific activities of purified XOR enzymes

The specific activities of XOR enzyme towards xanthine have been calculated using the urate assay (Section 3.2.4.2). Total protein was estimated using the Bradford method (Section 3.2.3). Values are means ± SD (n = 3).

A graphical representation of these activities is shown below:

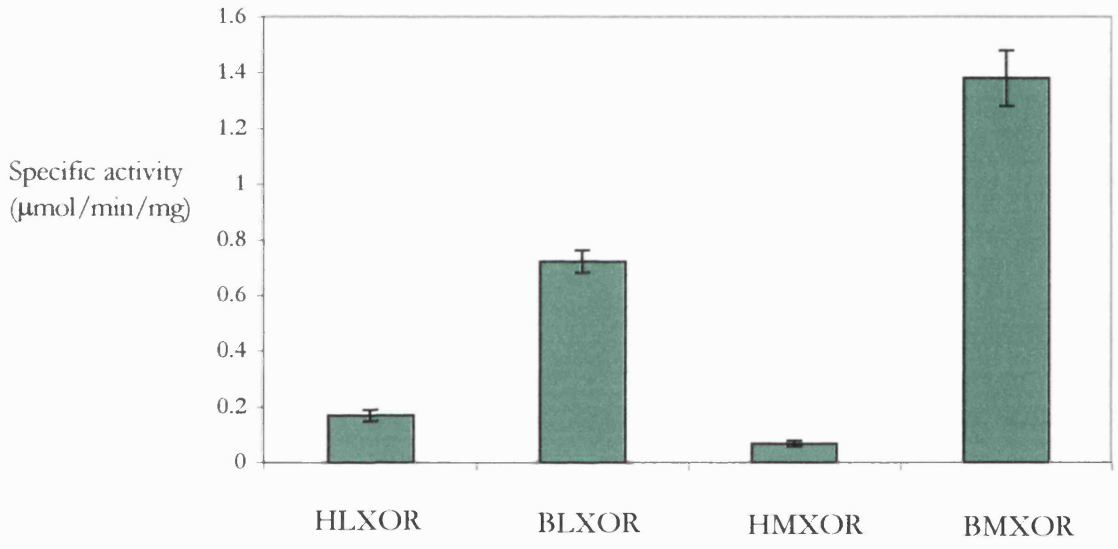
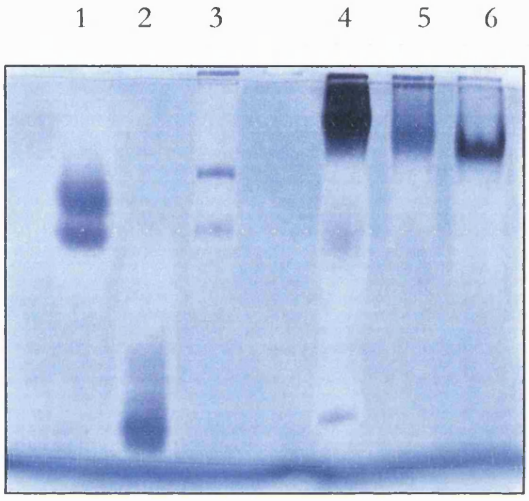


Figure 9.3.1 Graph showing specific activities of purified XOR from human liver, bovine liver, human milk and bovine milk

Specific activities have been calculated as in Table 9.3.1. Values are means ± SD (n = 3).

9.3.2 Activity staining

HLXOR, HMXOR and BMXOR were subjected to native-PAGE and activity staining (Section 9.2.2.1). The results are shown below:



6 % Native-PAGE

1 – carbonic anhydrase

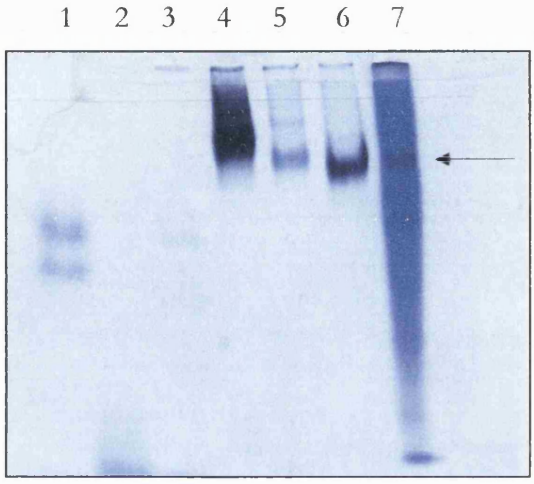
2 – chicken egg albumin

3 – urease

4 – BMXOR

5 – HMXOR

6 – HLXOR



6 % Native-PAGE

1 – carbonic anhydrase

2 – chicken egg albumin

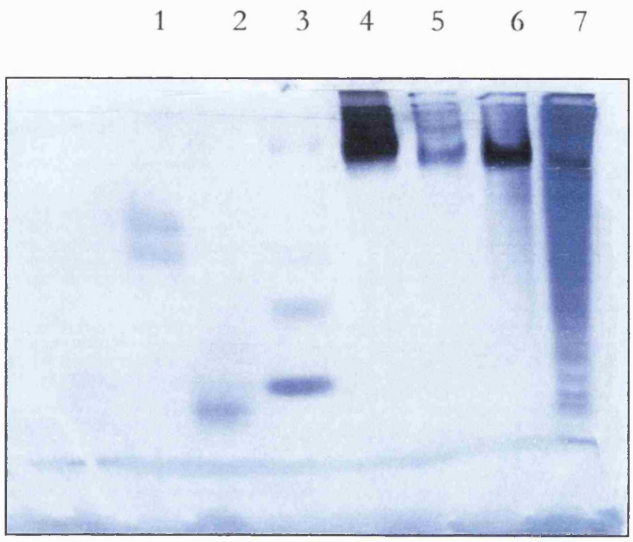
3 – urease

4 – BMXOR

5 – HMXOR

6 – HLXOR

7 – whole liver homogenate



7 % Native-PAGE

1 – α-lactalbumin

2 – carbonic anhydrase

3 – chicken egg albumin

4 – BMXOR

5 – HMXOR

6 – HLXOR

7 – whole liver homogenate

Figure 9.3.2 **6 % and 7 % native-PAGE of HLXOR, HMXOR and BMXOR**

Purified XOR enzymes (7.5 μg) were run alongside markers.

9.3.3 XOR quantification by ELISA

ELISA quantification was performed (Section 3.2.5.1). Whole bovine milk, whole human milk, human liver homogenate and bovine liver homogenate were assayed.

Assay	ELISA estimation of XOR protein concentration (ng HXO/mg total protein)	Specific activity (μmol/min/mg XOR protein)
Bovine milk	37 ± 11	9.2 ± 2
Human milk	25 ± 9	0.8 ± 0.06
Bovine liver homogenate	182 ± 47	5.8 ± 1.2
Human liver homogenate	204 ± 35	1.6 ± 0.6

Table 9.3.2 XOR quantification by ELISA

XOR protein concentration was calculated by ELISA. Total protein was estimated using the Bradford assay (Section 3.2.3). Specific activity was calculated using the urate assay (Section 3.2.4.2). Values are means ± SD (n = 3).

9.3.4 Specific activities of primary biliary cirrhotic liver and normal liver

Specific activities were measured in the homogenates (Section 8.2.2.1) from these two liver samples, using the more sensitive pterin assay.

Liver sample homogenate	Specific activity (pmol/min/mg total protein)	% Oxidase
PBC liver	0.65 ± 0.1	65
Normal liver	0.16 ± 0.01	52

Table 9.3.3 Specific activities of primary biliary cirrhotic liver and normal liver

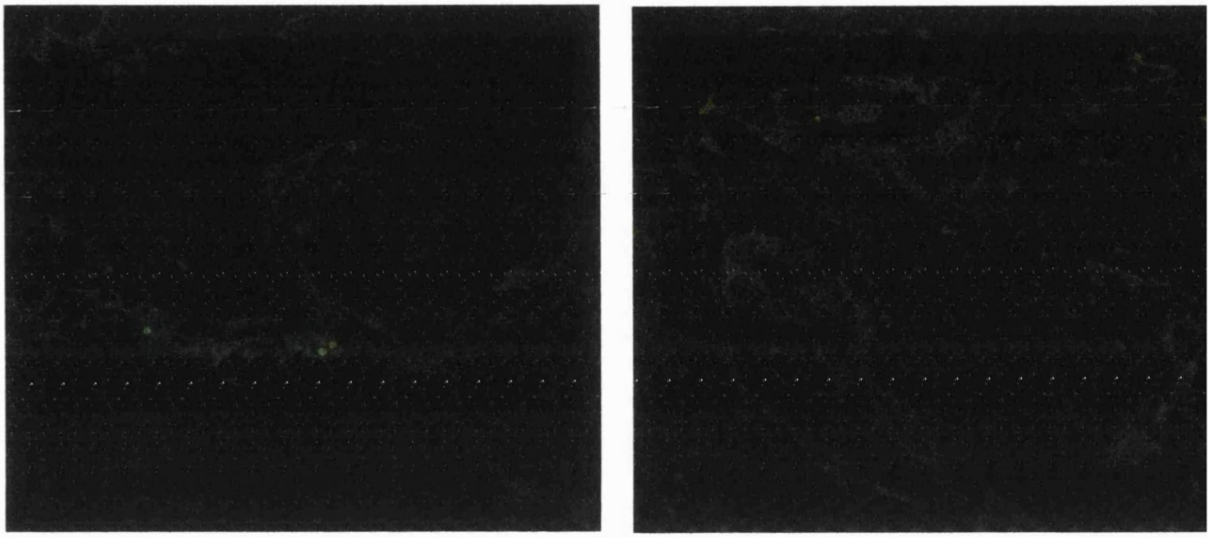
Pterin assay was conducted (Section 3.2.4.1) and total protein was estimated using the Bradford method (Section 3.2.3). Values are means ± SD (n = 3).

9.3.5 Immunohistochemical staining of XOR in liver

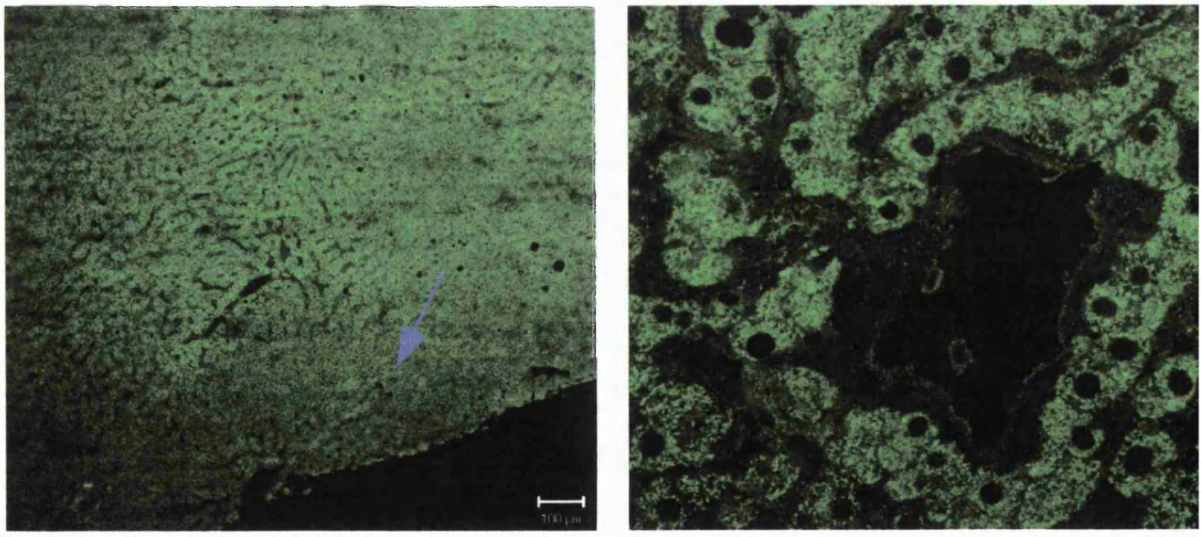
1D9D1 monoclonal antibody was used for the purpose of immunohistochemical localisation of XOR in liver sections. Previous studies within our laboratory had obtained successful results using this antibody to localise XOR in cell lines (personal communication, C. Hoare).

Immunohistochemistry was carried out as described in Section 9.2.2.3. The negative control liver sections were incubated with blocking solution and then with secondary antibody only to ensure that there were no trace amounts of XOR in the blocking solution, or non-specific binding occurring.

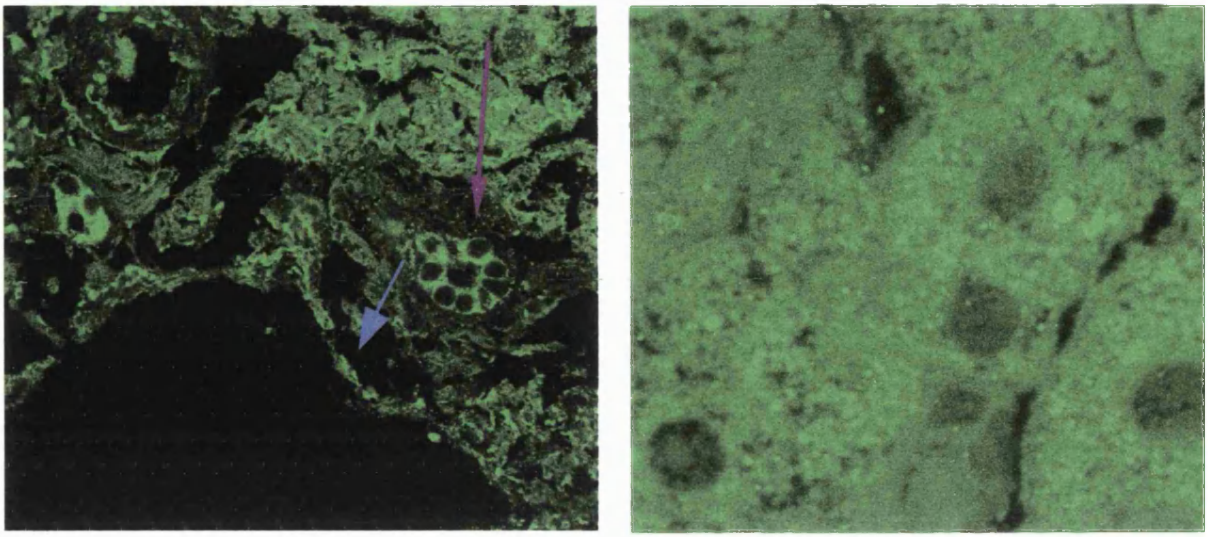
9.3.5.1 Normal human liver



A B



C D

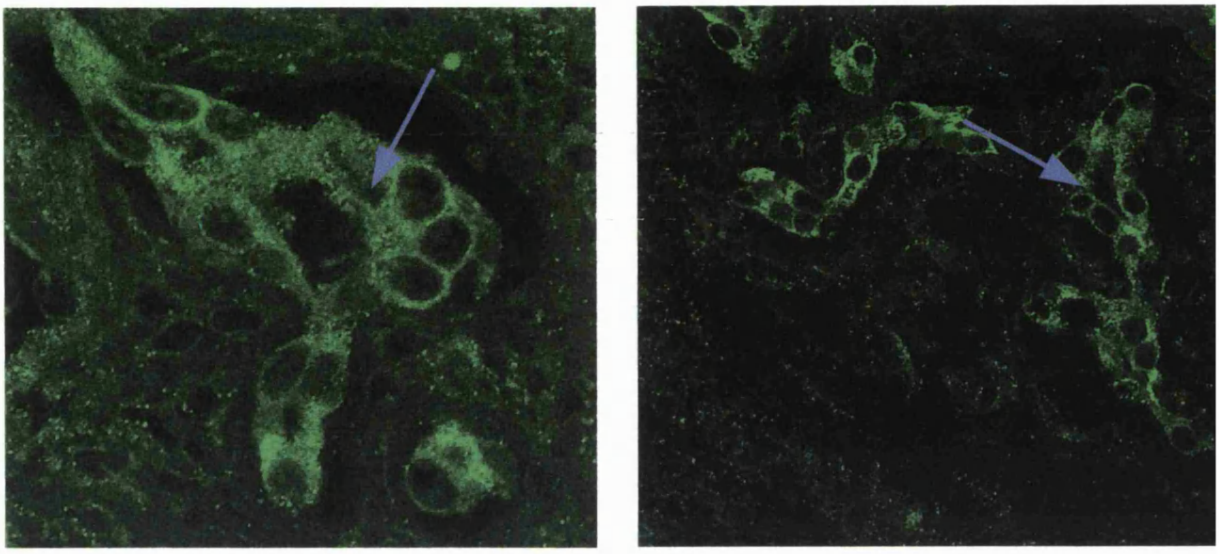


E **F**

Distribution of XOR in normal liver sections

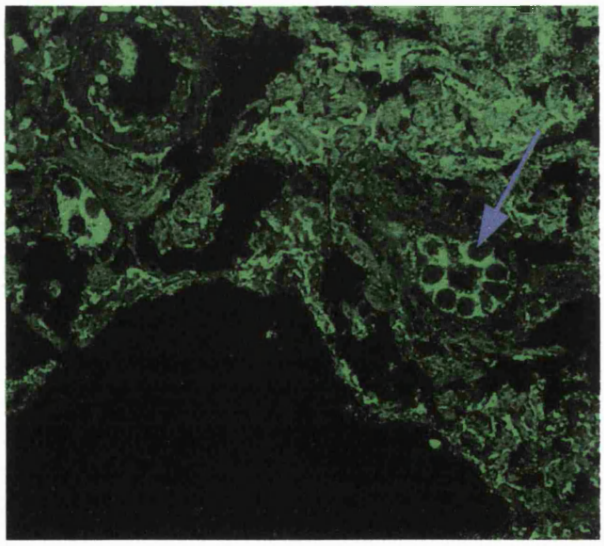
The negative controls (**A**) and (**B**), where the liver sections were incubated with secondary antibody only, yielded no staining of XOR. Slides (**C**) – (**F**) were stained for XOR. There is an uneven pattern of positive staining in the hepatocytes (**C**), where the arrow highlights hepatocytes with weaker XOR expression. (**D**) and (**F**) illustrate punctate or granular staining of hepatocytes. In (**E**), the Kupffer cells appear to be both positive (highlighted by short arrow), and negative. Also in (**E**), there is apparent a bile ductule, exhibiting strong staining of XOR (highlighted by long arrow).

9.3.5.2 Primary biliary cirrhotic (PBC) human liver



A B

Atypical bile ductule conformation in primary biliary cirrhotic liver sections



C

Typical bile ductule in normal human liver

(**A**) and (**B**) show atypical bile ductules in primary biliary cirrhosis (highlighted by arrows), where an elongated configuration is observed. (**C**) represents normal liver and here the typical ductule shows distinct lumen and cell borders. Both PBC and normal liver exhibit very strong staining of XOR in the ductules.

9.3.6 Crystallisation of HLXOR

9.3.6.1 Hampton crystal screen

Crystallisation of immunopurified HLXOR was attempted, as described in Section 9.2.2.4. The Hampton Research Crystal Screen was used as an initial set of conditions and all subsequent crystallisation experiments were carried out at 20 °C.

The table below illustrates the conditions that produced positive results:

Crystal Screen	Result
1. 30 % PEG 4000, 0.1 M Tris-HCl pH 8.5, 0.2 M magnesium chloride	crystalline precipitate
2. 20 % iso-propanol, 0.1 M Na-acetate pH 4.6, 0.2 M calcium chloride	microcrystals
3. 18 % PEG 8000, 0.1 M Na-cacodylate pH 6.5, 0.2 M Zinc acetate	crystalline precipitate

Table 9.3.4 Positive results obtained with the Hampton Crystal Screen

9.3.6.2 Further screens

Further screens were designed, based on the second combination of precipitant, buffer and salt (Table 9.3.4) in the Hampton screen, yielding microcrystals. This condition included 20 % iso-propanol, 0.1 M Na-acetate, 0.2 M calcium chloride, pH 4.6. All screens also contained 10 mM DTT, which has been found to be a prerequisite for the crystallisation of HMXOR (Pearson, A., Ph.D. Thesis, 2001).

	12 % iso-propanol	14 % iso-propanol	15% iso-propanol	17% iso-propanol
pH 4.5	0.15 M $CaCl_2$ 0.1 M Na-acetate **precipitate**	0.2 M $CaCl_2$ 0.1 M Na-acetate **negative**	0.25 M $CaCl_2$ 0.1 M Na-acetate **needles**	0.2 M $CaCl_2$ 0.1 M Na-acetate **plate on edge**
pH 4.6	0.2 M $CaCl_2$ 0.1 M Na-acetate **precipitate**	0.15 M $CaCl_2$ 0.1 M Na-acetate **precipitate**	0.15 M $CaCl_2$ 0.1 M Na-acetate **small plates**	0.25 M $CaCl_2$ 0.1 M Na-acetate **single needle**
pH 4.7	0.25 M $CaCl_2$ 0.1 M Na-acetate **precipitate**	0.25 M $CaCl_2$ 0.1 M Na-acetate **precipitate**	0.2 M $CaCl_2$ 0.1 M Na-acetate **plates**	0.15 M $CaCl_2$ 0.1 M Na-acetate **starbursts**

Table 9.3.5 Further screens

Observations are indicated in the table.

9.3.6.3 Crystals

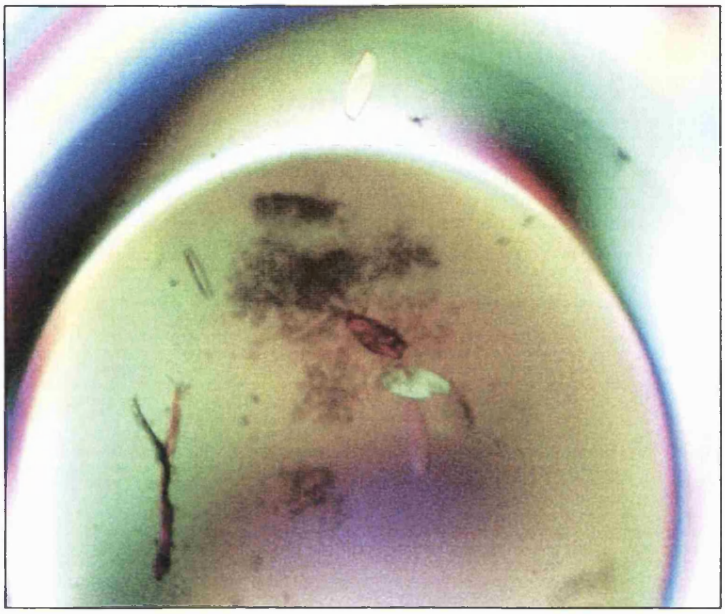
The following photographs illustrate the different types of crystals obtained. The pictures were taken with the use of a Leica microscope and a 35 mm film camera. Figs a) (i) & (ii), b), e) and f) are taken under polarised light.

a) 15 % iso-propanol, 0.1 M Na-acetate, 0.2 M $CaCl_2$, pH 4.7

(i)

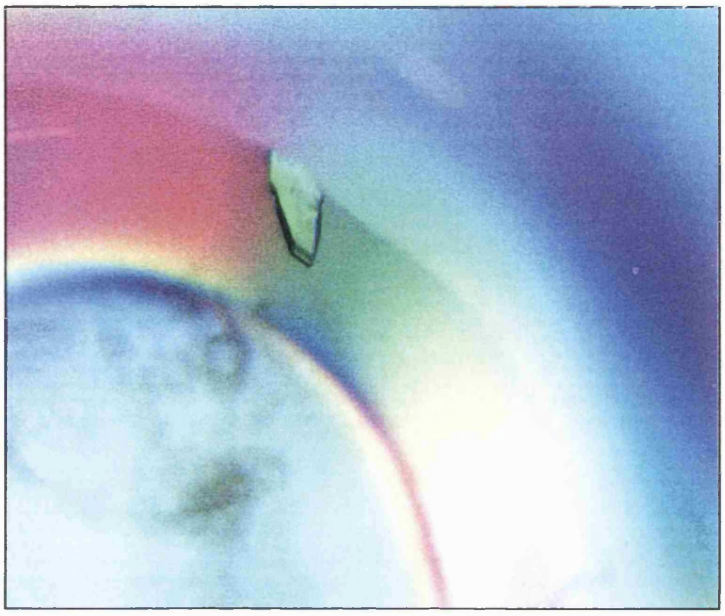
Three-dimensional crystal on edge of plate, approximately 0.13 mm in length

(ii)



Crystals in centre of oil droplet, approximately 0.13 mm in length

b) 17 % iso-propanol, 0.1 M Na-acetate, 0.2 M $CaCl_2$, pH 4.5

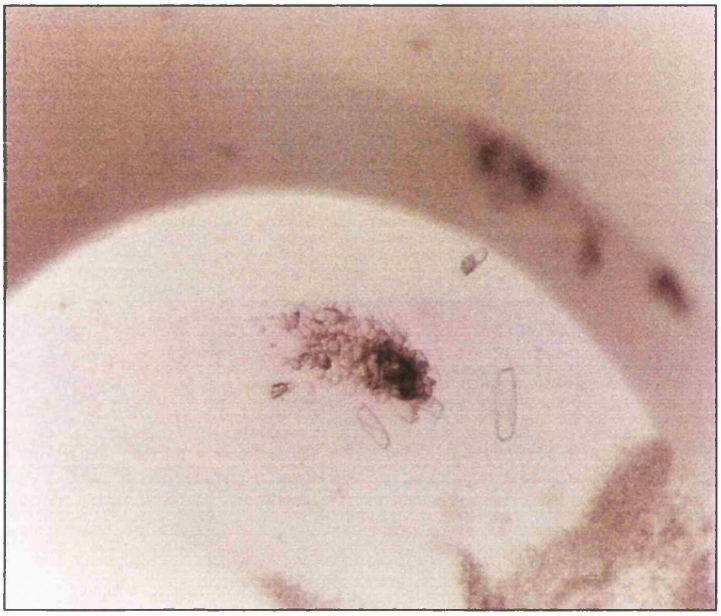


'Chunky plate' on edge of well, growing on surface of oil

c) 15 % iso-propanol, 0.1 M Na-acetate, 0.15 M $CaCl_2$, pH 4.6

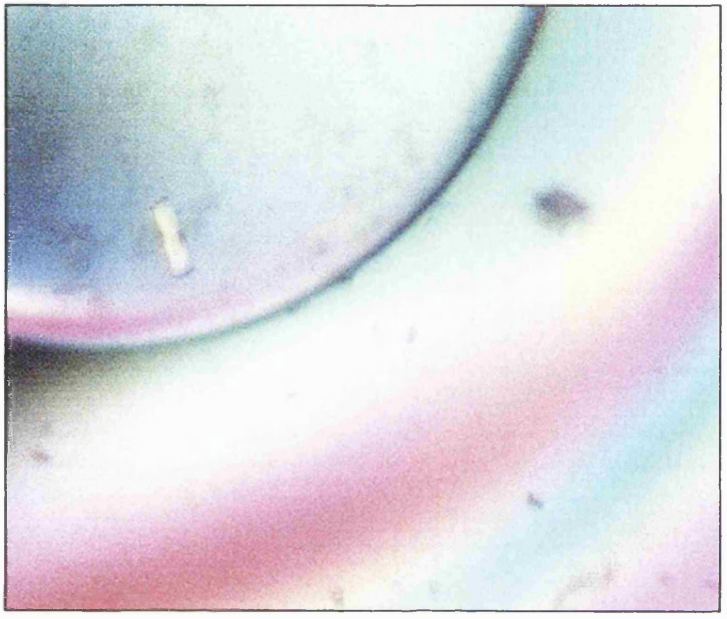
Very small 'plates' on top layer of oil

d) 15 % iso-propanol, 0.1 M Na-acetate, 0.25 M $CaCl_2$, pH 4.5



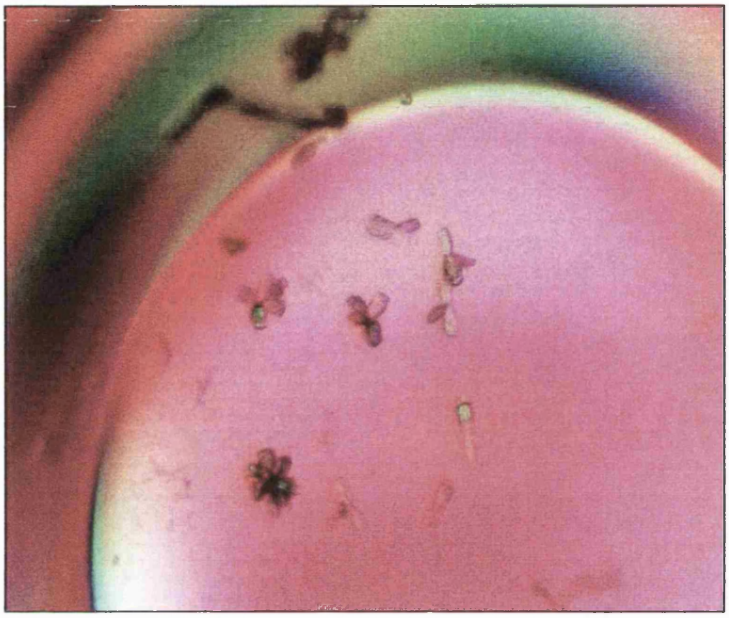
Single 'needles' on base of well

e) 17 % iso-propanol, 0.1 M Na-acetate, 0.25 M $CaCl_2$, pH 4.6



Single 'needle'

f) 17 % iso-propanol, 0.1 M Na-acetate, 0.15 M $CaCl_2$, pH 4.7



Several starbursts

It was attempted to solubilise a few of the smaller crystals and process them for Western blotting to ensure that they were indeed HLXOR. However, no result was evident on Western blotting, which was very likely due to insufficient protein present.

9.4 Discussion

In all mammals studied, liver and intestine have shown the highest XOR activity (Parks & Granger, 1986). This is also true for humans, although in this case activities are relatively low in all tissues. Particularly well studied have been human serum (Al-Khalidi *et al.*, 1965; Yamamoto *et al.* 1996) and heart (Eddy *et al.*, 1987; Muxfeldt & Schaper, 1987; Grum *et al.*, 1989; de Jong *et al.*, 1990), in which levels of XOR activity have been variously reported as being very low or undetectable. Low overall activity can reflect either low levels of enzyme protein and/or low specific activity of that protein. In comparisons of human and bovine milk, the very much lower XOR activity of the former has been shown to result from low specific activity of XOR protein, levels of which are similar in the two species (Abadeh *et al.*, 1992; Harrison, 1997; Godber, B., Ph.D. Thesis, 1998). Preliminary purification of XOR from human heart using immunoaffinity techniques, has indicated that a similar situation occurs in this tissue also (Abadeh *et al.*, 1993).

Purifications of XOR from human liver have been reported by Krenitsky *et al.* (1986) and Moriwaki *et al.* (1993), with widely differing specific activities towards xanthine of 1.8 μmol/min/mg and 0.96 x 10^{-3} μmol/min/mg respectively. The activity of the preparation obtained by Krenitsky *et al.* is comparable with that of XOR, purified from bovine milk (1.38 μmol/min/mg) in the present study (Section 9.3.1). The human liver enzyme, purified by immunoaffinity chromatography (Section 8.3.2.6) showed specific activity of only 0.17 μmol/min/mg, some ten-fold lower than the previous value reported by Krenitsky and colleagues. In contrast, this specific activity of the human

liver enzyme detailed in the present work is some 200-fold higher than the value reported by Moriwaki *et al.* above for their preparation from human liver.

There are several possible explanations for the discrepancies observed in specific activity of human liver XOR. Firstly, the immunoaffinity purification suffers from the disadvantage that successful elution conditions result in partial loss of FAD from XOR. This can, however, be largely rectified by incubation with FAD. Circulation of HMXOR on the column, followed by re-addition of FAD led to approximately 25 % loss of NADH oxidase activity (Section 8.3.2.3.4). Assuming that similar losses occurred in purification of HLXOR, we arrive at a specific activity of 0.23 μmol/min/mg, still only a fraction of the earlier value reported by Krenitsky.

It is important to take into consideration that Krenitsky *et al.* (1986) employed an affinity purification that depended on binding of active enzyme. Thus, Krenitsky used a guanine analogue, which, although much less studied, almost certainly is similarly selective. It is, accordingly, probable that, of the activity-based, affinity-purified preparations of HLXOR, only that described in this thesis is representative of enzyme in whole tissue. However, this cannot explain the significantly lower specific activity enzyme obtained by Moriwaki *et al.* (1993), who used a succession of affinity procedures including immobilised folate, which is well known to bind only XOR with active molybdenum sites (Ventom *et al.*, 1988).

HMXOR, prepared by affinity chromatography on heparin, had a specific activity of 0.068 μmol/min/mg (Section 9.3.1). This is comfortably within the range (0.03 – 0.1 μmol/ min/mg) quoted by Godber (Ph.D. Thesis, 1998) for very many preparations using the same procedure. Specific activities of heparin-affinity purified BMXOR reported by Godber (1.2 – 1.5 μmol/min/mg) are similarly in accord with that found in the present studies (1.38 μmol/min/mg). Based on average values, Godber estimated the BMXOR/HMXOR activity ratio to be approximately 20. A similar ratio was obtained for whole milk by comparison of overall enzymic activities and yields of XOR protein (Section 9.3.3).

If the specific activity of immunoaffinity-purified BLXOR is 'corrected' for FAD loss, as described above for HLXOR, we obtain 0.96 μmol/min/mg. Comparisons of XOR purified in this thesis then lead to the following conclusions. Bovine XOR shows generally higher specific activity than human enzyme, but the 20-fold difference in the milk XORs is not matched in liver, where the corresponding ratio is closer to 4.

The relatively low specific activities of the human enzymes are most probably attributable to the presence of inactive forms. BMXOR and HMXOR have been shown to contain approximately 40 % and 97 % respectively of demolybdo-enzyme, lacking molybdenum and possibly also the molybdopterin cofactor (Godber *et al.*, 1997; Bray *et al.*, 1999). Of the remaining molybdo-enzyme, some 40-50 % is likely to exist as inactive desulpho-form. Although the limited amounts of purified enzyme precluded direct estimation of the molybdenum content of HLXOR, its specific activity would be

consistent with a content of approximately 12 % molybdenum. BLXOR would, correspondingly, contain some 40 % molybdenum.

While the above discussion is reasonably self-consistent, it is not supported by ELISA data. Based on activity and ELISA data from whole tissue homogenates, Sarnesto *et al.* (1996) reported activity of 2.7-3.0 μmol/min/mg for HLXOR; an order of magnitude higher than that of my purified enzyme. Specific activities similarly derived in this thesis are also high (HLXOR, 1.6 μmol/min/mg; BLXOR, 5.8 μmol/min/mg; BMXOR, 9.2 μmol/min/mg; HMXOR, 0.8 μmol/min/mg). In view of the fact that the maximum specific activity of fully active BMXOR is calculated to be approximately 5 μmol/min/mg (Bray, 1975), these values are clearly inflated, possibly by about 6-fold. The most likely explanation lies in underestimation, by ELISA, of XOR protein, although the reason for this is not obvious. Whatever their relationship to true levels of XOR protein, relative ELISA values are of interest. These show XOR protein to be some 8-fold richer in human liver (204 ng/mg total protein) than in breast milk (25 ng/mg total protein), demonstrating that both higher levels of XOR protein and higher specific activity contribute to the greater activity of the enzyme in liver.

The activity staining results (Section 9.3.2) support the above. This was performed with the pure enzymes (human liver, human and bovine milk), which were subjected to electrophoresis under native conditions, and staining for activity with hypoxanthine and nitroblue tetrazolium. BMXOR stained most strongly, HLXOR stained second most strongly, and HMXOR produced the weakest staining, in accordance with their relative

specific activities. Staining of a faint band in human liver homogenate was also apparent.

Measurement of XOR specific activities in normal liver homogenate and primary biliary cirrhotic liver homogenate produced some interesting results. The more sensitive pterin assay method was used in this instance, due to the low activity in the homogenates. Primary biliary cirrhotic liver was over 4-fold richer in XOR activity than normal liver, and its homogenate contained a higher proportion of oxidase form enzyme than that from normal liver. Battelli *et al.* (2001) reported relatively high XO activity in the sera of patients with cholestasis, which is a feature of primary biliary cirrhosis. The accumulation of hydrophobic bile salts in this disorder is thought to lead to increased conversion of XDH to XO and the resulting ROS have been implicated in the oxidative damage of hepatic parenchymal cells, leading to liver fibrosis and cirrhosis (Schimpl *et al.*, 2000). During the early stages of injury, the raised levels of serum XO might be attributed to release of enzyme from liver cells. It is a possibility that upon injury, there is some upregulation of liver XO mRNA, possibly associated with interferon production (Terao *et al.*, 1992).

The immunolocalisation of XOR in liver sections was determined by using monoclonal anti-(human XOR) antibody 1D9D1, which displays crossreactivity with human liver XOR. Positive XOR expression was observed in all sections with very low background staining, as can be seen from the negative controls. In normal human liver, an uneven pattern of positive staining was found in the hepatocytes. While there appeared to be

little staining of sinusoidal endothelial cells, some Kupffer cells showed a positive response (Section 9.3.5.1, Figs (A)-(F)). An uneven pattern of staining in the hepatocytes, similar to that observed in the present study, has been reported by both Moriwaki *et al.* (1996b) and Linder *et al.* (1999). Both of these groups found a lobular gradient of XOR expression, with intense staining in the cytoplasm of the periportal hepatocytes, and weaker expression in the perivenular hepatocytes. It was not possible to determine, from the present limited study, whether the hepatocyte staining observed is periportal or pericentral, however. Linder *et al.* (1999) also reported strong staining in the Kupffer cells of approximately one-third of samples, in agreement with the present findings.

Of particular interest are the bile ducts, in which, in the present study, strong staining of XOR protein was observed. In primary biliary cirrhotic liver, atypical bile ductules were observed, with an elongated configuration, and these were also strongly stained. Normal ductules are characterised by well-formed lumina and distinct cell borders (Section 9.3.5.2, Fig ©). Atypical ductules have vague or no visible lumen and indistinct cell borders (Figs (A) & (B)). The occurrence of atypical ductules in primary biliary cirrhosis and other long-term cholestatic diseases has been well documented (Rubin *et al.*, 1965; Ludwig *et al.*, 1978; Nakanuma & Ohta, 1986; Van Eyken *et al.*, 1988). Primary biliary cirrhosis, in particular, is characterised by progressive destruction of normal bile ducts and proliferation of atypical ductules (Harada *et al.*, 1998), displaying an elongated or anastomosing configuration. The typical ductules were found to express complete biliary-type cytokeratins, while atypical ductules lacked complete biliary-type

cytokeratins and often connected with periportal hepatocytes. In the present work, bile ductules of both types, typical and atypical, were strongly stained and it seems likely that their proliferation could be a major factor in the observed increase in overall XOR activity observed in cirrhotic liver. The presence of XOR protein in bile ducts is in accord with the findings of Moriwaki *et al.* (1996b), but not of Linder *et al.* (1999), who failed to detect the enzyme in these structures.

It is possible that discrepancies arising from the results of immunolocalisation of XOR in the liver is due to the antibodies used, and also perhaps to the different procedures of detection. Linder *et al.* (2001) used a polyclonal anti-(HMXOR) antibody raised in rabbits in their study for the detection of XOR, whereas Moriwaki *et al.* (1996b) used a polyclonal anti-(HLXOR) antibody. This present study used a monoclonal anti-(HMXOR) antibody, with strong affinity towards both human milk and human liver enzyme. It is possible that the polyclonal anti-(HMXOR) antibody used by Linder *et al.* may not have been of sufficient sensitivity to detect XOR expression in the bile ducts, for example. Moriwaki *et al.* (1996b) used an antibody raised against purified human liver XOR. This may offer superior specificity to the antibody raised against human milk XOR used by Linder *et al.* (2001), particularly in the detection of human liver XOR. Such improved specificity might explain the positive XOR expression observed in the bile ducts in the study by Moriwaki *et al.* (1996b). The monoclonal anti-(HMXOR) antibody used in the present study may offer a specificity and sensitivity similar to the polyclonal antibody used by Moriwaki *et al.*, which might account for the similar findings.

Small crystals were obtained from purified HLXOR, using the microbatch under oil technique. Using the Hampton Research Crystal Screen conditions, a 'hit' was apparent in 20 % iso-propanol, 0.1 M sodium acetate, 0.2 M calcium chloride, pH 4.6. Further experiments were designed based on this condition, and several more 'hits' resulted. Crystals in the form of plates, and starbursts, were observed in both 15 and 17 % iso-propanol, 0.1 M sodium acetate, with varying concentrations of calcium chloride, and with pH between 4.5 and 4.7. The largest crystal was observed in 15 % iso-propanol, 0.1 M Na-acetate, 0.2 M calcium chloride, pH 4.7. All crystallisation conditions also contained 10 mM DTT, which has been found to be essential in the crystallisation of HMXOR. Unfortunately, due to the time constraints of this study, it was not possible to obtain diffraction data from these crystals.

Crystallisation of demolybdo human milk XOR has been recently reported (Pearson, A., Ph.D. Thesis, 2001). Hexagonal orthorhombic and trigonal HMXOR crystals were obtained (see Appendices). The human milk enzyme was found to crystallise under slightly different conditions, to that found in this study for liver, including 20-30% polyethylene glycol 4000, 0.1 M Na-acetate, Na-citrate or Tris, 0.15-0.25 M ammonium acetate or ammonium sulphate, pH 5-8, and 10 mM DTT.

10. Discussion

XOR from bovine milk, rat and chicken liver has been well characterised, however, up until recently, there has been little work carried out on the human enzyme, probably because of the abundance and ease of extraction of the enzyme from bovine milk. Interest in human XOR was greatly stimulated in 1981, when Granger *et al.* reported XDH to XO conversion in ischaemic tissues as a source of damaging ROS following reperfusion (Granger *et al.*, 1981). Since this finding, XOR-derived ROS have been implicated in a multitude of human diseases involving ischaemia-reperfusion injury (McCord *et al.*, 1985). Animal models have been used to investigate the role of XOR in reperfusion injury, and the XOR inhibitors, allopurinol and oxypurinol, as well as pre-treatment with tungsten, have been shown to prevent organ damage (Greene & Paller, 1992; Wiezorek *et al.*, 1994; Kurose & Granger, 1994). However, the interspecies variability in the tissue-specific expression of XOR activity does not allow conclusions from animal model experiments to be extrapolated to humans.

The pathological and physiological significance of XOR in humans is unclear. The low specific activity of the human milk enzyme for conventional reducing substrates remains intriguing. Furthermore, XOR expression and activity is largely confined to the liver and intestine, with specific activity of the enzyme in these organs reported to be higher than that in breast milk. These findings suggest a specialised distribution of XOR, which is not fully understood.

This thesis describes the purification and characterisation of XOR from human liver, with further investigation as to its localisation in this tissue. Previous purifications of XOR from human liver have been based on affinity (Krenitsky *et al.*, 1986; Moriwaki *et al.*, 1993) and immunoaffinity (Saksela *et al.*, 1999) methods.

The purification of XOR from bovine liver provided a starting point for the development of a suitable immunoaffinity purification procedure for the isolation of the enzyme from human liver. Initially, a procedure for the isolation of BLXOR based on non-immune affinity methods was developed, which provided a useful comparitor for purification using immunoaffinity procedures. BLXOR was affinity-purified to near homogeneity, with a molecular weight of approximately 135 kDa as judged by SDS-PAGE, using a combination of chromatographic methods, involving benzamidine-Sepharose, heparin-Sepharose, and HiTrap Q ion-exchange. The first of these, benzamidine-Sepharose chromatography, was based on a method described by McManaman *et al.* (1996) for the isolation of XOR from rat liver, while the latter two methods were derived from the purification of milk XOR used within the laboratory (Godber, B., Ph.D. Thesis, 1998). An overall purification factor of approximately 275-fold was obtained, which was higher than the 199-fold purification reported by McManaman *et al.* (1996), for rat liver XOR. The yield of BLXOR was comparable with that obtained by McManaman *et al.* Cabre & Canela (1986) reported the only published affinity purification of XOR from bovine liver. Their purification consisted of five chromatographic steps, together with heating and acetone precipitation (both of which were avoided in the present study, as potentially damaging to the enzyme). These

harsh methods possibly contributed to their finding of multiple bands on SDS-PAGE analysis of their preparation, indicating significant degradation. Despite this apparent heterogeneity, an absorption curve was acquired from their BLXOR, from which a PFR of 5.64 was calculated. In the present work, it was not possible to obtain a UV-visible scan resembling XOR from the affinity-purified BLXOR, indicating possible contamination with other proteins.

Rabbit polyclonal anti-(HMXOR) antibodies were affinity-purified, for use in immunoaffinity purification. Western blotting of bovine liver homogenate with these polyclonal antibodies showed a band approximating to 150 kDa. A three-step purification method was devised, consisting of ammonium sulphate precipitation, Protein A-Sepharose and immunoaffinity chromatography. This resulted in an almost homogeneous preparation of BLXOR as judged by SDS-PAGE. Two bands were evident on SDS-PAGE gel, corresponding to molecular weights of 134 kDa and 50 kDa. A monoclonal anti-HMXOR antibody reacted with both bands in Western blotting. The major band at 134 kDa was comparable to the band at 135 kDa observed previously, with affinity-purified BLXOR. BLXOR purified by Cabre & Canela (1986) also displayed a major band with molecular weight of 135 kDa, similar to that obtained here.

Immunoaffinity-purified BLXOR showed a UV-visible spectrum characteristic of XOR from bovine and human milk. A PFR of 5.75 was calculated from the scan, indicative of reasonably pure XOR, with the typical range for highly purified XOR from milk

between 5.2 and 5.7. This is comparable to the PFR of 5.62 reported by Cabre and Canela (1986) for their BLXOR preparation. The yield of immunoaffinity-purified BLXOR obtained was ten-fold higher than that obtained in my non-immune affinity purification. The rapid processing, higher yield, and the relative purity of enzyme obtained, as indicated by the PFR, give immunoaffinity purification distinct advantages over the non-immune purification procedure.

The polyclonal immunoaffinity method used in the BLXOR purification was further optimised for the isolation of XOR from human liver. Both polyclonal and monoclonal anti-(HMXOR) antibodies were investigated. HLXOR purified by polyclonal antibodies produced three bands on SDS-PAGE gel, corresponding to approximately 150 kDa, 135 kDa and 50 kDa. The 135 kDa band represented the major band, whilst the minor band was at 150 kDa. Western blotting using a monoclonal anti-(HMXOR) antibody identified the 150 kDa and 135 kDa bands as XOR. N-terminal sequence analysis of the 135 kDa and 50 kDa bands revealed matches of the 135 kDa band with human XOR, and the lower 50 kDa band with heavy chain immunoglobulin. Such immunoglobulin may have been present from the beginning of the purification, perhaps existing as an immune complex with XOR, or have arisen as a result of leaching from the immunoaffinity column matrix into the eluate. Saksela *et al.* (1999), also using polyclonal immunoaffinity purification, reported a HLXOR preparation displaying a similar pattern on SDS-PAGE gel, with bands at 150 kDa, 130 kDa and 85 kDa.

The use of monoclonal antibodies in the isolation of HLXOR required Western blot analysis in order to identify a clone with suitable specificity towards the enzyme. In fact, only one of the three clones tested displayed immunoreactivity (1D9D1) towards HLXOR. The corresponding antibody was accordingly selected for use in purification. Employment of monoclonal antibodies required modifications of the polyclonal immunoaffinity method: CNBr-activated Sepharose 4B was used as an alternative coupling medium to Protein A-Sepharose, and glycine was substituted as an eluant in the place of diethylamine. A final step involving a circulation on Protein L-Sepharose was introduced in order to remove any contaminating immunoglobulin (see above). Monoclonal immunoaffinity-purified HLXOR displayed two bands on SDS-PAGE gel, a major band at approximately 155 kDa and a minor band at 135 kDa. Western blotting using polyclonal affinity-purified anti-(HMXOR) identified both bands as XOR. This preparation appeared to be less proteolysed than HLXOR from polyclonal immunoaffinity chromatography, in which the major band corresponded to the lower molecular weight band of 135 kDa, with the minor band at 150 kDa.

The specific activities towards xanthine and the yields of HLXOR purified by the two methods were very similar. The monoclonal immunoaffinity method offered an improvement in purification over the polyclonal immunoaffinity method, as judged by SDS-PAGE and native gel electrophoresis. Unfortunately, neither method led to UV-visible spectra with features characteristic of XOR. As previously mentioned, this may be due to some structural damage to the enzyme, altering the UV-visible properties. It is unlikely that the absorption spectrum for human liver XOR should differ from those

of XOR enzymes isolated from other sources. For example, the absorption spectrum of mouse liver XOR (Carpani *et al.*, 1990) bears strong similarities to that of other XOR preparations from rat liver (Suleiman & Stevens, 1987) and bovine milk (Massey *et al.*, 1969).

It is evident from this work that differences exist in the apparent molecular weights of the various XOR preparations, as judged by SDS-PAGE gels. It is well established in our laboratory that freshly-prepared BMXOR and HMXOR each show a strong band at approximately 150 kDa. A weaker band at 135 kDa, attributed to proteolysis, may or may not be present initially, but increases in intensity with age of the preparation. The major band of HMXOR consistently runs slightly slower than does that of BMXOR; a fact that is difficult to explain in view of the close similarity of the primary structures of these enzymes (Section 8.4). These findings are corroborated in the present work, which shows that HLXOR also follows the above pattern. BLXOR, in contrast, whether prepared by immune or non-immune affinity chromatography, showed only one high molecular weight band at 134/135 kDa. Cabre and Canela (1986) similarly reported a band of 135 kDa on SDS-PAGE (although further lower molecular weight bands were also present) and a corresponding molecular weight for the non-denatured enzyme from gel-filtration analysis. The obvious conclusion is that the 135 kDa bovine liver XOR band represents a proteolysis product, equivalent to those of BMXOR, HMXOR and HLXOR. It is, nevertheless, a strong, clean band without evidence of a higher molecular weight companion. It is possible, although improbable, that bovine liver contains a particularly active proteolytic enzyme, that is less active or absent in

equivalent homogenates from other tissues. Alternatively, BLXOR could actually be smaller than other mammalian XORs. Clearly, further investigation is desirable.

Despite the apparent species-specific variations in molecular weight of XOR, the enzymes appear to share similar patterns of degradation. Sarnesto *et al.* (1996) observed that the cleavage of purified XOR and XOR in tissue homogenates is not random, with similarly sized fragments consistently apparent on SDS-PAGE and Western blots. This indicates conservation of cleavage sites within the amino acid sequences of XOR enzymes. In human liver XDH (Ichida *et al.*, 1993), the tryptic cleavage sites of the rat liver enzyme (Amaya *et al.*, 1990) are conserved (Sarnesto *et al.*, 1996). Krenitsky *et al.* (1986) reported the presence of five bands on SDS-PAGE analysis of their purified HLXOR. Sarnesto *et al.* (1996) reported the presence of multiple bands in human liver homogenised in the presence of protease inhibitors, upon Western blotting, and suggested that this may reflect the existence of XOR in partially-proteolysed form in intact liver. HLXOR purified in the present work shows minimal proteolysis, with only two bands on SDS-PAGE gel. A similar pattern of bands was also observed in liver homogenate. This is not consistent with the proposition of the presence of *in vivo* proteolysed HLXOR (Sarnesto *et al.*, 1996).

Aldehyde oxidase (AO), another molybdoflavoprotein, shares both structural and catalytic similarities with XOR. It has been suggested that the two enzymes arose from a common progenitor by gene duplication and subsequent genetic modification, a view supported by genetic data (Krenitsky, 1978). They are both homodimers with a

molecular weight of 300 kDa. Furthermore, the subunits from the two proteins each contain one atom of molybdenum, one molecule of FAD and four Fe/S groups, and thus have very similar absorption spectra. It was clearly important to ascertain, during the course of this study, that enzyme purified was XOR and not AO. Purified preparations of XOR were routinely assayed for AO contamination by using 1-methylnicotinamide, a compound readily oxidised by AO, but not by XOR (Krenitsky *et al.*, 1972). No activity was detected in the purified preparations, although activity was present in whole bovine and human liver homogenate. AO also has a similar distribution to XOR in mammals, with high levels of enzyme present in the small intestine and liver (Krenitsky *et al.*, 1974) leading to the proposal that the two enzymes together may constitute a protective barrier in these organs, capable of detoxification of mainly nitrogen-containing heterocycles (Krenitsky, 1978). However, this proposal is less attractive in the case of humans, where the levels of enzyme activity attributed to AO are very low (Krenitsky *et al.*, 1974). XOR activity levels, on the other hand, have been reported to be in the same order of magnitude, albeit to some extent lower, to levels in other mammalian species (Parks & Granger, 1986).

Affinity-purified rabbit polyclonal and mouse monoclonal anti-(HMXOR) antibodies have been characterised during the course of this study. Both types of antibody cross-reacted with XOR from bovine milk, human milk, bovine liver, and human liver, recognising minor proteolysis bands on Western blots. No cross-reactivity has been observed between polyclonal anti-(XOR) antibodies and AO protein in an antibody specificity study carried out by Moriwaki *et al.* (1996a), so it is likely that the polyclonal

antibodies used in this study were not reactive with AO. The monoclonal antibody 1D9D1 was the only one from three clones tested that showed interaction with HLXOR. This antibody also displayed the strongest avidity towards the immunogen HMXOR, although weaker reaction was observed between this antibody and the bovine liver and milk XOR enzymes. As total homogenate was used in Western blotting to determine the specificity of the antibody to HLXOR, it should be mentioned that the proportion of XOR protein of total protein in homogenate is very low, and therefore the negative results obtained during blotting of homogenate with the other two monoclonal antibody clones may simply indicate lack of sensitivity of the Western blotting method, rather than lack of specificity of the antibody. The specificity of 1D9D1 for HLXOR was also confirmed by immunoprecipitation of the enzyme from human liver homogenate. SDS-PAGE of the immunoprecipitate showed a band corresponding to XOR. In view of this, the antibody 1D9D1 was selected for subsequent immunoaffinity purification, and for use in later immunolocalisation studies.

Perhaps the most noteworthy point, arising from this study, is the surprisingly low specific activity of purified HLXOR. An early report describing the first purification of XOR from post-mortem liver revealed this enzyme to possess a specific activity towards purine substrates similar to that of BMXOR, although considerable differences were noted between the bovine and human enzymes in their activity towards nucleoside substrates (Krenitsky *et al.*, 1986). At around the same time, a report described a similarly high catalytic activity towards conventional reducing substrates in the liver and small intestine, with activity low or undetectable in other organs (Parks & Granger,

1986). A few years later, XOR was purified from human milk, and shown to have surprising properties (Abadeh *et al.*, 1992; Harrison, 1997; Godber *et al.*, 1997). Activity of HMXOR towards xanthine was found to represent only 1-2 % that of the bovine milk or rat liver enzymes, although the NADH oxidase activity, which generates hydrogen peroxide and superoxide anion at significant rates, was shown to be similar to that of the bovine milk enzyme (Sanders *et al.*, 1997). The low activity of the human milk enzyme has been explained in terms of exceptionally low molybdenum content. Purified HMXOR appears to contain at least 95 % demolybdo-enzyme, as apposed to 30-40 % for purified BMXOR. Moreover, desulpho-XOR makes up at least 40 % of molybdenum-containing HMXOR (Harrison, 1997). The low specific activity of XOR in human milk is not unique. Human heart XOR was affinity-purified and shown to yield enzyme with a similarly low specific activity (Abadeh *et al.*, 1993). Based on the above information, a proposal that two types of XOR existed within the human was made, comprising of 'low' activity enzyme in milk and heart, and 'high' activity enzyme (similar to that of BMXOR) in liver and intestine (Abadeh *et al.*, 1992; Harrison, 1997). Low activity has also recently been identified in cultured human epithelial cells, by ELISA and activity assays (Page *et al.*, 1998).

Many subsequent biochemical studies have confirmed the existence of significant XOR activity in human liver and small intestine with none or negligible XOR activity detected in other organs (Watts *et al.*, 1965; Eddy *et al.*, 1987; Grum *et al.*, 1989; Smolenski *et al.*, 1989; de Jong *et al.*, 1990; Kooij *et al.*, 1992). Values for the specific activities of bovine and human liver XOR, based on the purifications carried out in the present work, are

discussed in Chapter 9. BLXOR was found to have specific activity lower than, but nonetheless of the same order of magnitude as that of BMXOR. The specific activity of HLXOR, however, was surprisingly low. In contrast to the working hypothesis, noted above, of 'high activity' XOR (i.e. 'BMXOR-like') in human liver and intestine, HLXOR showed specific activity only some 4-fold higher than that of HMXOR. The idea of 'bovine-like' specific activity in HLXOR was initially derived from the purification of Krenitsky *et al.* (1986). However, as discussed in Chapter 9, this purification involved affinity chromatographic procedures that selected for active enzyme only. My immunoaffinity-purified preparation is much more likely to be representative of the mix of active and inactive forms of XOR present *in vivo*. In summary, we are left with a pattern of generally high activity XOR in bovine tissues (liver and milk), and much lower activity enzyme in humans, although HLXOR is nevertheless more active than is HMXOR. The commonly observed higher activity of XOR in liver (and probably intestine) compared with other human tissues reflects both relative specific activities and levels of XOR protein, although the latter is most likely to be more important.

The different specific activities of HMXOR and BMXOR have been shown to reflect their relative contents of demolybdo-XOR (Godber *et al.*, 1997; Bray *et al.*, 1999). Although the low amounts of purified enzyme precluded direct analysis, it is reasonable to assume that the specific activities of HLXOR and BLXOR are similarly dependent on molybdenum content. It is, however, by no means clear what mechanism determines these differences. The primary sequences of HLXOR (Ichida *et al.*, 1993) and BMXOR (Berglund *et al.*, 1996) are 90 % homologous, while those of HLXOR and

HMXOR (Pearson, A., Ph.D. Thesis, 2001) are essentially identical. It seems that differences in content of molybdo-XOR are most likely to be controlled by regulation of molybdenum-incorporating enzymes in the host cell.

There is strong evidence that the specific activity of human XOR is subject to regulation within the cell. Thus, Brown *et al.* (1995) showed wide variation of XOR activity in breast milk after parturition, while the amount of XOR protein remained essentially constant. Inflammatory cytokines such as TNF-α and IL-1 have been found to cause the upregulation and post-translational activation of XOR in a human mammary gland cell line, implicating the enzyme as a potential mediator in inflammation (Page *et al.*, 1998). Again, upregulation of XOR activity was not found to be matched by an increase in XOR protein. As noted above, such apparently post-translational activation of XOR could be brought about by regulation of the enzymic machinery that controls molybdenum incorporation, although this would seem to be a cumbersome mechanism. Simpler, at least in concept, would be conversion of inactive desulpho-XOR to its active sulpho form. Evidence for enzymic catalysis of this conversion has been demonstrated *in vitro* (Nishino *et al.*, 1983; Nishino, 1986). However, the extent of activation possible by these means is limited. Desulpho-XOR generally makes up 40-50 % of molybdo enzyme. In the case of HMXOR, with, for example, 3 % theoretical molybdenum content, the activity, could, at most, be doubled in this way. [Brown *et al.* (1995) observed up to 50-fold activation of XOR activity in post-partum breast milk]. An intriguing, further possibility involves phosphorylation of XOR protein, a potential activation mechanism that has been identified in hypoxia (Kayyali *et al.*, 2001). The

above discussion of post-translational activation mechanisms excludes upregulation of the enzyme by *de novo* synthesis, which undoubtedly also occurs, particularly in response to both cytokines (Page *et al.*, 1995) and hormones (McManaman *et al.*, 2000). Whether or not complex mechanisms of regulation of XOR activity are unique to man, as apposed to for example, rodents or cows (tissues of which appear to show uniformly high activity) is far from clear. The idea of evolution driven sophisticated regulation in humans is attractive, but entirely speculative at this stage.

It was outside the scope of the present study to measure specific activity in homogenates from a range of normal liver tissues and so explore whether the low specific activity observed in the liver used in this study simply reflects individual variations. Such variations have indeed been noted (Guerciolini *et al.*, 1991; Kooij *et al.*, 1992). The specific activity of XOR was measured in primary biliary cirrhotic liver homogenate and found to be over 4-fold higher in XOR activity than that from normal liver. Although there are currently no reports directly concerning enzyme activity in primary biliary cirrhosis, there are some indications that XOR activity is higher in cholestasis, a related feature of primary biliary cirrhosis (Schimpl *et al.*, 2000; Batelli *et al.*, 2001). Certainly serum XOR levels are known to be elevated as a consequence of a range of liver disorders (Ramboer *et al.*, 1972; Bhide *et al.*, 1974; Wolko & Krawczynski, 1974; Batelli *et al.*, 2001). Interestingly, there is some evidence of an increased nitric oxide production in primary biliary cirrhosis (Battista *et al.*, 2001). The enhanced nitric oxide production was suggested to be related to the development of strong inflammation, and also to neutrophil activation. If XOR is upregulated in the

pathogenesis of this disorder, it might prove to be involved in production of both nitric oxide and other free radicals, both of which have been implicated in the pathogenesis of primary biliary cirrhosis (Ono *et al.*, 1991).

Immunohistochemical localisation of XOR in liver sections in the present work has been carried out with the same mouse monoclonal anti-(HMXOR) antibody as that used in immunoaffinity purification: 1D9D1. This antibody has also been used within our group in studies of the subcellular localisation of XOR in human endothelial and epithelial cell lines, and has proved to be more effective that affinity-purified polyclonal anti-(HXOR) antibodies. Preliminary results have shown some evidence of vesicular export of XOR to defined patches of endothelial cell membrane (Hoare, C., personal communication). Previous conclusions concerning the immunohistochemical localisation of XOR in the liver have been conflicting, which may reflect the different specificities and avidities of the anti-(XOR) antibodies employed. For example, Linder *et al.* (1999) used a polyclonal anti-(HMXOR) antibody to localise XOR in a range of human tissues, and commented on the greater sensitivity of this antibody over the mouse monoclonal anti-(HMXOR) used by Hellsten-Westing (1993) for localisation in human cardiac and skeletal muscle. However, a monoclonal antibody is generally expected to have greater specificity over a polyclonal antibody.

In the present work, in normal human liver, XOR expression was detected in the hepatocytes, bile ductules, and some Kupffer cells. An uneven pattern of staining was observed in the hepatocytes, in line with previous observations (Moriwaki *et al.*, 1996b;

Linder *et al.*, 1999). Similar positive staining in Kupffer cells has been reported by Linder *et al.* (1999), who found this to be the case in approximately one-third of their liver samples.

In primary biliary cirrhotic liver, as also seen in normal liver, strong XOR expression was observed in the bile ductules and the hepatocytes. Furthermore, atypical bile ductule configuration was noted, a finding well documented in primary biliary cirrhosis and other long-term cholestatic diseases, and previously reported by Harada *et al.* (1998). It was not possible, however, using these methods of immunolocalisation, which were non-quantitative, to elucidate whether there was increased XOR expression in the primary biliary cirrhotic liver sections, as opposed to the normal liver, which would support the higher specific activity findings, and the theory of upregulated XOR mRNA expression, perhaps by cytokines, in this liver disease.

The crystallisation of HLXOR has been described in this work. Small crystals of HLXOR were obtained, using the Hampton Research Crystal Screen set of conditions. Crystals of HLXOR provide an exciting basis for future work, leading to the elucidation of the structure of this enzyme, which can be compared to the solved crystal structures of the bovine milk and human milk enzymes.

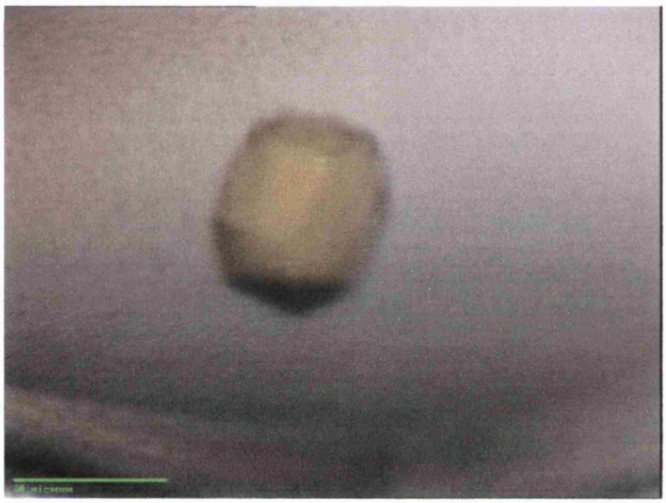
In summary, this work presents some interesting observations regarding the specific activity of HLXOR. The low specific activity of the liver enzyme is in contrast to the generally high pattern of activity reported previously for enzyme from this tissue. The specific activity of HLXOR is also lower than that of the bovine milk and corresponding liver enzyme. It would be worthwhile to further explore the basis of the differing kinetics, using a range of substrates in addition to pterin and xanthine, between these purified enzymes isolated from different species.

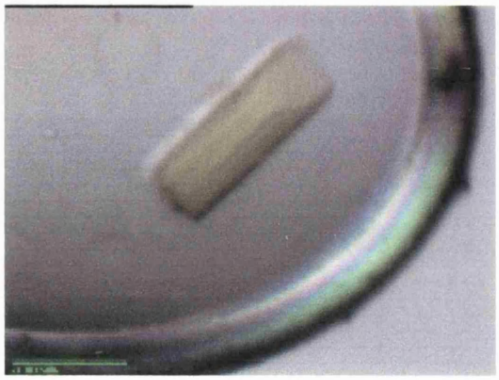
Additional further work might involve investigation into the molecular basis of the specific activity of HLXOR, by assessing the molybdenum content of this enzyme, and contrasting it with that of the lower activity HMXOR. In addition, the Fe/S composition can be examined, with X-ray analysis of the three-dimensional crystal structure of the enzyme; suitable conditions of crystallisation for the liver enzyme have been established in this study. Furthermore, it will be interesting to establish the extent of individual variation of human liver XOR, with particular relation to diseased liver, and this will help contribute to our understanding of the role of the enzyme in this tissue.

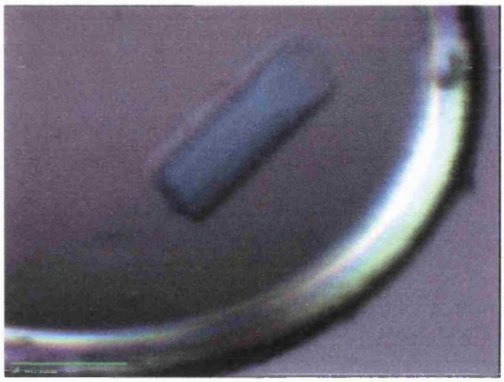
Appendices

Crystals of human milk xanthine oxidoreductase (HMXOR)

Photographs kindly donated by A. Pearson (Pearson, A., Ph.D. Thesis, 2001)







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